ENDOCRINOLOGY

VOLUME 60

JULY–DECEMBER 1956

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PUBLISHED FOR
THE ENDOCRINE SOCIETY

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George Banta Company, Inc.
Menasha, Wisconsin

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ENDOCRINOLOGY

VOLUME 60 JANUARY, 1957 NUMBER 1

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THE BLOOD CALCIUM RESPONSE OF THE CHICKEN TO PARATHYROID EXTRACTS[1]

DONALD POLIN,[2] PAUL D. STURKIE AND WALTER HUNSAKER

Laboratory of Avian Physiology, Rutgers University, New Brunswick, New Jersey

THE data concerning the effects of mammalian parathyroid extracts (PTH) on blood calcium of birds are inconsistent and inadequate. A few investigators have reported that these extracts increased blood calcium in the pigeon (1), and in the laying hen (2, 3), but an even larger number have reported negative results: i.e.; chickens (4, 5, 6, 7, 8, 9); ducks (10). Yet certain findings suggest that the bird, particularly the chicken, does respond to PTH injections.

The case of the laying hen with idiopathic hypoparathyroidism that responded to PTH treatment (11) is not to be overlooked. More important, Polin and Sturkie (13) observed a remission of symptoms in parathyroidectomized chickens injected with PTH, but the effects of the injection persisted for only 8–10 hours. This prompted further investigation into the problem of the PTH effect on blood calcium of the chicken.

Our observations and the few positive results reported by other investigators (1, 3) suggested that blood samples should be removed within a few hours after the injection of the hormone, rather than at the 18 and 24 hours as employed by other investigators.

Data are presented which show that the response of the chick to graded doses of PTH is of relatively short duration.

EXPERIMENTAL PROCEDURE

Cross-bred and White Leghorn chickens were maintained in batteries and fed a commercial mash and permitted water, *ad libitum*. The cocks and male chicks were starved 18–24 hours prior to injection of parathyroid extract (Eli Lilly) and during the 7-hour

Received March 14, 1956.

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University—The State University of New Jersey, Department of Poultry Husbandry. Supported by a grant from Limestone Products Corporation of America.

[2] Present address: Merck Institute for Therapeutic Research, Rahway, N. J.

experimental period following the injection. The hens were not starved because starvation markedly decreases their blood calcium levels (13). The chicks were 5–7 weeks-of-age, the cocks were 4–8 months-of-age, and the laying hens were in their first or second year of production (9–24 months of age).

Fifty units of PTH per injection were administered subcutaneously in the pectoral area; thus, a chick receiving a total of 150 U. was injected three times at three different sites. The control animals received injections of physiological saline.

A 3 ml. blood sample was removed by heart puncture from the chick, but from the brachial vein of the cock and laying hen. The various times at which the blood samples were withdrawn are noted in the charts and tables of this paper. The plasma, later obtained from the blood withdrawn into a heparinized syringe, was analyzed for calcium by the method of Elliott (14), slightly modified. The procedure was as follows: To ½ ml. of plasma are added 8.5 ml. of distilled H_2O and 1.0 ml. of N NaOH. Two drops of calcium indicator[3] (one capsule in 60 ml. of distilled H_2O) are added, and the resulting cherry-red color is titrated to an orchid-purple end-point by continuous titration with a solution of 0.001N disodium ethylenediaminetetraacetic acid. This procedure has a coefficient of variation of approximately 4%, and an expected recovery error of 2%.

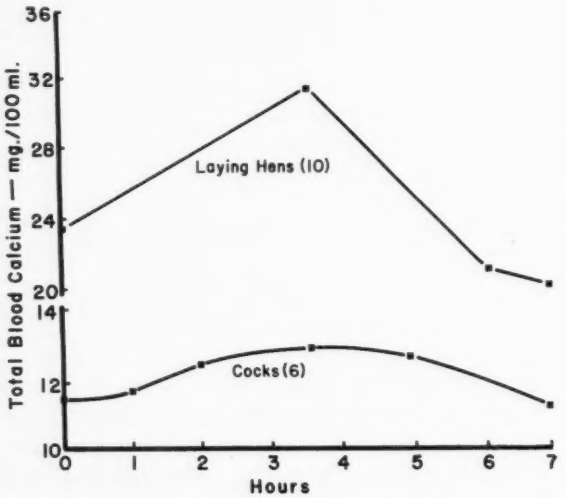

FIG. 1. Calcium response curve of cocks (16–32 weeks of age) and laying hens (1 and 2 years of age) to parathyroid extracts—100 U. subcutaneously injected.

RESULTS AND DISCUSSION

Normal cocks responded to PTH after a subcutaneous injection of 100 U. per bird, with a maximum rise in total calcium occurring 3–4 hours after injection (Fig. 1). This response was further substantiated when 13 other cocks, also injected with 100 U. and sampled at the expected time of maximum rise in plasma calcium showed a significant increment of 2.1 ± 0.39 mg./100 ml. over a control group ($P < 0.01$).

[3] Obtained from Hagan Corporation, Pittsburgh, Pa.

PTH produced a marked rise in plasma calcium of laying hens $3\frac{1}{2}$ hours after injection, but calcium levels were nearly normal 6 hours after the injection (Fig. 1). Some hens exhibited an increase in blood calcium by as much as 15 mg./100 ml., while others showed no response at the $3\frac{1}{2}$ hour-blood sample. The latter hens may have responded before or after the sampling time. The results of Knowles, Hart, and Halpin (3) who found the response to parathyroid extract in normal laying hens varied and often marked, are substantiated. The synergistic effect of PTH and estrogen on blood calcium, as previously suggested (3) is also confirmed. This is evident from the fact that the same dose of 100 U./bird produced in the cock a mean increment in plasma calcium of 2.1 mg./100 ml., as compared to the 7.6 mg./100 ml. for the laying hen.

Log-dose Response to Parathyroid Extracts (PTH)

The uniformity in the response of the cock to PTH suggested the use of the chicken not under the influence of estrogen (i.e., chick or cock) as a possible assay animal for PTH.

Chicks, 5 and 7 weeks old, were sampled at 3–4 hours after PTH injection, the time at which the cock was found to respond maximally. Two trials were conducted to obtain log-dose response curves for PTH. The trials were performed with the assumption that the time-dose response curve in the chick was similar to that obtained in the cock. The same chicks were used in Trials 1 (5 weeks-of-age) and 2 (7 weeks-of-age). They were randomly selected for each trial. Dosages of 50, 100, 150 and 200 U. of parathyroid extract were injected. The decreased number of birds used in Trial 2 reflects the losses that occurred as a result of the blood sampling by heart stab.

The slopes for the two regression lines calculated from the data of Table 1 are for Trial 1, $Y=8.07+2.68X$, and for Trial 2, $Y=4.90+3.98X$, where $X=$log units of PTH and $Y=$plasma calcium in mg./100 ml. The observed values of both trials are plotted in Figure 2 along with the calculated log-dose response regression curves. The data of the two trials show good agreement with the exception of that for the 50 unit dose (Table 1).

TABLE 1. EFFECT OF PARATHYROID EXTRACTS (PTH), INJECTED SUBCUTANEOUSLY INTO 5-WEEK- (TRIAL 1) AND 7-WEEK- (TRIAL 2) OLD CHICKS. BLOOD SAMPLES REMOVED 3–4 HOURS AFTER INJECTION

Treatment	No. of birds		Calcium, mg./100 ml.		Mean increment in calcium over control	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Control—NaCl	7	7	11.00	10.94	0	0
50 units PTH	7	7	12.83	11.65	1.83	0.71
100 units PTH	7	7	13.19	12.97	2.19	2.03
150 units PTH	7	6	13.57	13.38	2.57	2.44
200 units PTH	7	2	14.64	14.34	3.64	3.40

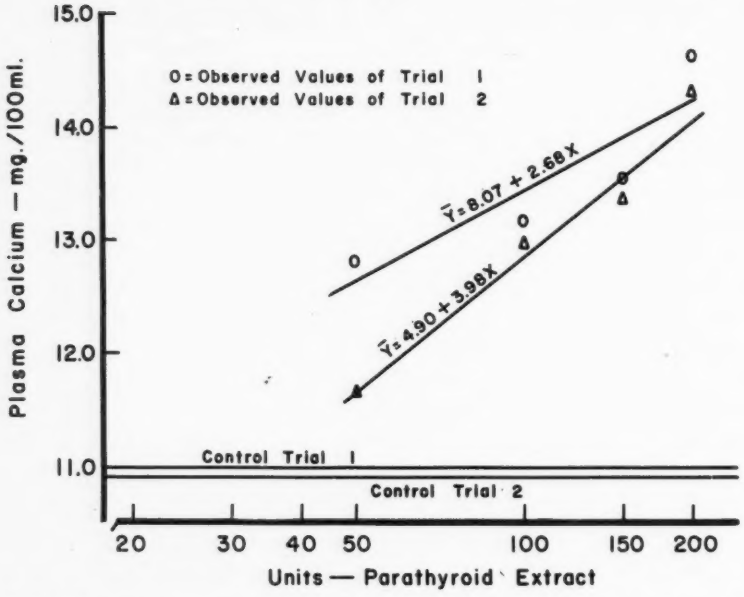

FIG. 2. Log-dose response curves for parathyroid extracts, subcutaneously injected into 5-week- and 7-week-old chicks. Blood samples removed 3–4 hours after injection.

An analysis of the data (15) indicated that the curves were linear (Table 2) and that the slopes in the two trials were not significantly different (Table 3).

The preliminary data obtained from the two trials suggest the chick as a suitable animal for biological assays of parathyroid extracts. The increments in plasma calcium obtained in the chicks receiving the 100 unit dose (2.19 and 2.03 mg./100 ml.) correspond to that obtained in the cock with similar treatment (2.1 mg./100 ml.) and indicate reproducibility of the response to PTH, particularly at this dose. The response of the chick to PTH was rapid. Blood samples were easily obtained from the 5- and 7-week-old chicks used in this experiment. An even younger chick could probably be used, the limiting factor being the ability to obtain sufficient

TABLE 2. ANALYSIS OF VARIANCE OF DATA FROM THE DOSE RESPONSE TRIALS

Source of variation	Trial 1		Trial 2	
	d.f.	Mean square	d.f.	Mean square
Doses	3	4.29*	3	5.57**
Slope of line, b	1	10.30**	1	15.74**
Scatter of doses about line	2	1.29	2	.49
Remainder	24	1.43	18	.99
	$s = 1.20$		$s = .99$	

* $P \leqq .05$.
** $P \leqq .01$.

TABLE 3. REGRESSION ANALYSIS OF DATA FROM DOSE RESPONSE TRIALS

	d.f.	Correlation coef.	Regress. coef.
Trial 1	3	.90*	2.68
Trial 2	3	.97**	3.98
Avg. within Trials	6	.92**	3.22

Analysis of Variance

	d.f.	Mean square	F
Between Reg. Coef.	1	.98	1.1
Dev. from Individ. Regress.	4	.89	

* $P \leqq .05$.
** $P \leqq .01$.

blood for the calcium analysis. The chick was used twice. It is an economical laboratory animal to maintain and is expendable. All of these characteristics are desired in an assay procedure, and suggest the use of the chick, provided the data presented are confirmed by further trial.

SUMMARY

Laying hens and cocks respond maximally to parathyroid extracts, 3–4 hours after subcutaneous injection of 100 units. The laying hen showed a greater blood calcium response than the cock or chick. Log-dose response data for the chick are presented and the response was linear over the range of 50–200 units that were employed. One hundred units of parathyroid extract, subcutaneously injected into 5- and 7- week-old chicks and into mature cocks, increased plasma calcium an average of 2.1 mg./100 ml., 3–4 hours after injection.

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A COMPARISON OF METHODS FOR EXTRACTING PITUITARY ANTIDIURETIC SUBSTANCE FROM URINE

D. C. JESSUP, N. B. G. TAYLOR AND R. L. NOBLE

Department of Medical Research, University of Western Ontario, London, Ontario, Canada

URINARY excretion of an antidiuretic substance, presumably of posterior lobe origin, has been studied by numerous workers. Such a substance has been reported to be present in a variety of conditions such as dehydration (1), toxemia of pregnancy (2, 3), and hypertension (4). The most frequently used method of extracting the antidiuretic substance has been dialysis either with a cellophane membrane or collodion (1, 5, 6, 7). Dialysis methods of extraction have been criticized on the grounds of inefficiency (8, 9), and variability (10).

In 1939, Noble, Rindernecht and Williams (11) proposed a method of extracting posterior lobe antidiuretic hormone ADH from urine, where the activity is adsorbed on zinc ferrocyanide and eluted with a solution of ammonia in ethyl alcohol. This method of extraction has been used in this laboratory for several years and has been found to recover consistently from 70% to 90% of the antidiuretic activity of ADH in urine. The chief drawback of this method is that the final extracts have a high total solid content and, in addition, contain a substance or substances which augment the antidiuretic effect of the extract when assayed by subcutaneous injection. In addition a strongly pressor substance, not vasopressin, is present, which interferes with the use of intravenous assay methods for ADH (8, 11).

In 1949 Grollman and Woods (12) devised a method for extracting ADH wherein the activity was adsorbed on activated charcoal. This method appeared to produce extracts of lower total solid content than the zinc ferrocyanide adsorption method and was reported to allow a high rate of recovery.

The present investigation is concerned with comparing the latter two methods of extraction in an effort to determine the efficiency of each to recover ADH from urine.

METHODS

(1) *Extraction by Adsorption on Zinc Ferrocyanide*

Extraction Procedure: Freshly collected urine from non-smoking, normally hydrated laboratory members was prepared by adjusting it to pH 5.0 and specific gravity of 1.010.

Received April 20, 1956.

To each 100 ml. of urine was added 1.0 ml. of 2N zinc sulphate. Next was added, dropwise with vigorous stirring, 1.0 ml. of 2.25N potassium ferrocyanide per 100 ml. of urine. A flocculent white precipitate was produced. This mixture was cooled in the refrigerator, then centrifuged for 15 minutes at 1500 r.p.m. in a centrifuge with a head 33.0 cm. in diameter. The supernatant was discarded. The precipitate was washed three times with a solution of 1% ammonia in 80% ethyl alcohol in volumes of 25 ml. per 100 ml. Between elutions the mixture was centrifuged. The eluates were pooled and retained. The aggregate volume of eluates was then reduced under a vacuum of 25 mm. Hg pressure. During the reduction to dryness the flask containing the solution was kept in a water bath, the temperature of which was less than 50° C. The dry residue was then suspended in 9.0 ml. of distilled water and some of the remaining contaminants precipitated with 10 volumes of absolute ethyl alcohol. After centrifugation the alcohol was evaporated to dryness under vacuum as described above. The dried residue was made up to a volume suitable for bioassay. The most satisfactory results were obtained when the extract was concentrated to 5% of the original urine volume.

The antidiuretic hormone in different known concentrations was added to aliquots of a large volume of normal human urine. Each aliquot was subjected, separately, to extraction by adsorption on zinc ferrocyanide. Control extracts were prepared by the same method and from the same urine volume. ADH was then added to the extracts after extraction instead of to the urine as described above.

(2) *Extraction by Adsorption on Activated Charcoal*

Extraction Procedure: The original procedure described by Grollman and Woods was followed except that the urine was adjusted to a specific gravity of 1.010 before extraction. The adjustment was made to ensure a relatively constant total solid concentration of the urine as well as for comparison with the other method which calls for a fixed urine density. The urine was broght to a pH of 4.5 to 5.0 after adjustment of the specific gravity. To each 100 ml. of urine was added 1 gm. of activated powdered charcoal (Darco, Grade G-60). This mixture was stirred at intervals for several hours then placed in the refrigerator at 4° C. overnight. Next day the supernatant urine was discarded and the charcoal washed in a Buchner funnel with a few mls. of water and transfererd to a centrifuge tube when dry. Glacial acetic acid, 5.0 ml. per gm. of charcoal, was added and the mixture stirred. After centrifugation the supernatant was removed and to it was added 10 volumes of a mixture containing equal parts of petroleum ether (boiling range 39.4°–54.9°) and absolute ethyl alcohol. In most cases the mixture was placed in the refrigerator overnight to aid precipitation. The mixture was then centrifuged and the precipitate retained and placed in a vacuum desiccator at room temperature to remove the remaining organic solvents. The dry residue was then made up to a concentration suitable for bioassay of antidiuretic activity. A concentration equal to 5% of the original urine volume was used.

Known concentrations of ADH were added to urine and subjected to exctraction by adsorption on charcoal. Bio-assays of these extracts and those produced by adsorption on zinc ferrocyanide were performed simultaneously.

(3) *Method of Assay*

Extracts were assayed by the method of Burn (1937) (13). Male rats of the Sprague-Dawley strain weighing 150–225 gm. were used in groups of 4 animals per cage. Food was taken away from the rats for 18 hours immediately prior to the assay but they were allowed free access to water. At the end of the fasting period the rats were given, by stomach tube, warmed water in a volume equal to 5% of their body weights. They received subcutaneous injections immediately after receiving the water load and were placed in cages over collecting funnels. Each cage contained 4 rats. Urine was collected in

graduated cylinders and the volume of urine was noted at 15 minute intervals throughout the assay.

A modification of the Burn method of assay was used in determining the 50% excretion time. In the present investigation the 50% time was taken to be that time at which 50% of the total volume of urine was excreted. Total excretion was considered to have occurred if more the 75% of the administered water was excreted and when the volume of urine excreted during a half-hour period diminished to less than 1.5 ml.

RESULTS

(1) *Control Extracts:* Extracts of normal human urine were prepared by each method and assayed for antidiuretic activity. Six separate extracts were prepared by each method and each extract assayed on 2 groups of rats on six different occasions. The dose of extract was 0.5 ml. per 100 gm. body weight. The zinc ferrocyanide extracts produced a mean 50% time of 97.2 min. ±2.1 (12); charcoal extracts produced a mean 50% time of 100 min. ±2.6 (12). The mean 50% time for the non-injected controls was 85 min. ±2.7 (16). An increase in the 50% time over that produced by control extracts is indicative of antidiuretic activity providing that all extracts are of the same concentration.

Determination of the total solid contents of the two extracts showed the zinc ferrocyanide extract to have a much higher total solid content (310 mg. per 100 ml. of urine, S.G. 1.010) than the charcoal extract (6.5 mg. per 100 ml. of urine, S.G. 1.010). These figures apply to extracts which have been concentrated to 5% of the original urine volume.

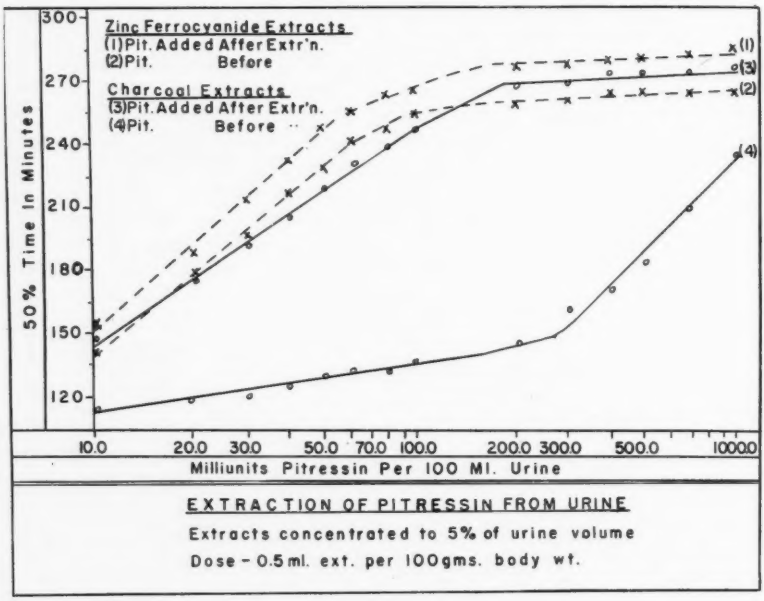

FIG. 1

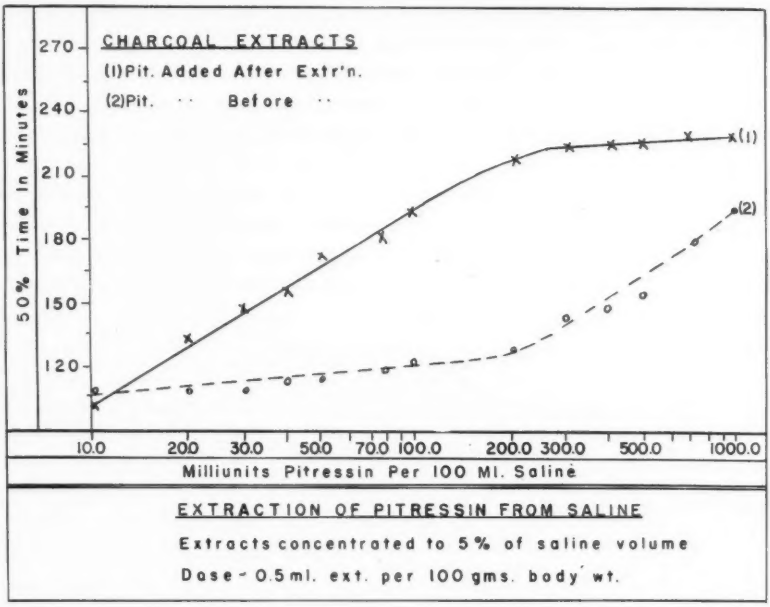

FIG. 2

(2) *Extraction of ADH from Urine:* The two extraction procedures were compared to determine their relative merits with regard to the recovery of ADH added to normal human urine. Figure 1 illustrates the results obtained for the assay of extracts having Pitressin (Parke, Davis) added after extraction and of extracts of urine to which Pitressin was added before extraction. Differences in the 50% time of any one concentration of Pitressin are due to the loss of activity during extraction. Hence it will be noted that the recovery rate for the zinc ferrocyanide method of extraction is approximately 80% for concentrations of ADH between 10.0 MU. and 100 MU. per 100 ml. of urine. For the charcoal adsorption method a negligible amount of Pitressin is recovered when the concentration is less than 200 MU. per 100 ml. of urine. For concentrations greater than 200 MU. per 100 ml. of urine the recovery rate is approximately 10%.

At least four separate extracts were prepared by each method for each concentration of Pitressin shown in Figure 1. Each extract was assayed in duplicate.

(3) *Extraction of ADH from Sodium Chloride Solution:* The recovery of ADH from urine by charcoal adsorption was considerably less than that reported by Grollman and Woods for the recovery of ADH from 0.9% sodium chloride solution. To determine where the discrepancy in the results lay, the experiment was repeated using 0.9% saline instead of urine. Different concentrations of Pitressin were added to saline before extraction and to the extracts after extraction. Figure 2 shows the results obtained

from the assay of these charcoal extracts of saline. Almost no ADH was recovered when the concentration was less than 200 mU. per 100 ml. of saline. At concentrations greater than 200 mU. per 100 ml. of saline between 7% and 10% of the antidiuretic activity was recovered. Four separate extracts were prepared and assayed in duplicate for each concentration of ADH.

It is not possible to extract posterior lobe antidiuretic hormone from 0.9% saline using the zinc ferrocyanide method of extraction as described, even when the concentration was at 1.0 unit per 100 ml. of saline. Apparently the urinary constituents are necessary to enable the extraction procedure to be carried out satisfactorily.

DISCUSSION

The results of this investigation show that by adsorption onto zinc ferrocyanide approximately 80% of the antidiuretic activity of added posterior lobe hormone may be recovered from urine. This confirms the findings of Noble *et al.* (11) and Taylor (14). The recovery rate obtained for the extraction of ADH from urine and from 0.9% sodium chloride solution by charcoal adsorption is less than 10%. This result differs greatly from the 90% to 100% recovery rate obtained by Grollman and Woods, whose procedure was followed in detail.

The possible explanation of the discrepancy between these results and those of Grollman and Woods would seem to be related to their dose response curve. The response to different concentrations of ADH obtained by them was the same as the response obtained in this investigation for ADH solutions that were 10 times as dilute. That is, when they assayed ADH in saline at concentrations of 20.0, 30.0, 50.0 and 100.0 milliunits, their assay results corresponded to those reported here for the assay of 2.0, 3.0, 5.0 and 10.0 milliunits respectively. Curve (1) of Figure 2 shows the response obtained for the assay of concentrations of Pitressin ranging from 10.0 mU. to 1000.0 mU. per 100 ml. of urine; the same curve could be used to show the response obtained by Grollman and Woods for concentrations ranging from 100.0 mU. to 10,000 mU. per 100 ml. of urine. Consequently, if the assay results of charcoal extracts used in this study were assessed from the Grollman and Woods' dose response curve, the recovery rate would be 10 times as great as was obtained from the standard dose response curve presented here. This dose response curve is essentially the same as reported by other investigators (10, 11, 13, 15).

The loss of activity during extraction by charcoal adsorption was shown when zinc ferrocyanide extracts, prepared from the different steps of the charcoal adsorption method, were assayed. These assays gave some indication of the relative distribution of the lost antidiuretic activity. They showed that approximately 25% of the total activity was not adsorbed on the charcoal but lost in the supernatant urine. About 50% of the total activity remained in the acid eluate after precipitation. Since 10% of the

total activity was recovered in the eluate, the remaining 15% was probably not eluted from the charcoal.

Extraction by adsorption on zinc ferrocyanide is, in most instances, the extraction method of choice because of its relatively high and constant recovery rate. Also, the augmentation of antidiuretic activity produced by zinc ferrocyanide extracts on subcutaneous injection has been found to be constant. This is illustrated by the low standard errors determined for the mean 50% times produced by extracts containing varying concentrations of ADH. The augmentation factor is therefore an advantage in that it increases the sensitivity of the assay method, allowing for the detection of lower concentrations of antidiuretic activity. For quantitative methods it is obviously essential to add known amounts of ADH to control urine extracts containing the augmenting factors.

The chief fault found with the method of extraction by adsorption on zinc ferrocyanide was the high total solid content of the extract. This hypertonicity necessitates the dilution of zinc ferrocyanide extracts to not less than 5% of the original urine volume. If the extracts are concentrated more than this, for example to 1% of the original urine volume, the high total solid content interferes with the assay. Inhibition of diuresis results when hydrated rats are injected with solutions having high osmotic concentrations (10, 16, 17). This inhibition of diuresis makes it difficult to distinguish between the effect of ADH and the high total solid content of the extract.

Dilution of the extracts overcomes the difficulty of hypertonicity. Since the augmenting factors present in zinc ferrocyanide extracts increase the sensitivity of the assay, the dilution of the extracts and hence of the antidiuretic activity, is offset.

It was felt that, since the extracts prepared by charcoal adsorption had a very low total solid content even when highly concentrated, this method of extraction would be more valuable than the zinc ferrocyanide adsorption method. However, this advantage gained by using the charcoal extract is overshadowed by the low rates of recovery.

Considering the results obtained for the assay of control extracts, it will be noted that there is a difference of about 15 minutes between the 50% times resulting from the assay of the urine extracts and the 50% times produced by non-injected controls. This difference is constant and significant. The slight inhibition of diuresis resulting from the injection of urine extracts is believed to be due to the presence of an antidiuretic substance in the urine of normal healthy humans. We have been able to show that such a substance does exist and that it can be extracted by either of the extraction methods described in this paper (14).

SUMMARY

Two methods of extracting added posterior lobe antidiuretic substance from urine have been studied. Each method employs adsorption, one on

activated charcoal, the other on zinc ferrocyanide, for recovering ADH. The method employing zinc ferrocyanide adsorption recovers 80% of the ADH added to urine. The method employing charcoal as the adsorbent recovers less than 10% of the antidiuretic substance.

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THYROID IN B-12 DEFICIENT CHICK EMBRYOS[1]

T. M. FERGUSON, R. H. RIGDON AND J. R. COUCH

Departments of Biochemistry and Nutrition and Poultry Husbandry, Agricultural and Mechanical College of Texas, College Station, Texas and the Laboratory of Experimental Pathology, University of Texas Medical Branch, Galveston

AN ENLARGED thyroid gland was observed in B-12 deficient chick embryos during the study of the pathologic changes associated with this deficiency (1, 2). The extreme enlargement of the gland was not apparent when vitamin B-12 was injected either parenterally into the hens maintained on the low B-12 diet or when injected directly into the eggs prior to incubation (1). The pathologic changes in the thyroid of these B-12 deficient embryos are now reported.

MATERIAL AND METHODS

The deficient embryos were obtained from hens fed a synthetic-type diet without vitamin B-12 (1). The normal embryos were from hens fed a practical-type diet adequate in respect to all known nutrients (3). The vitamin B-12 content of the egg yolk was determined at intervals according to the method described by Skeggs and co-workers (4). The size of the thyroids was determined by measuring the gland of 59 embryos in the two largest diameters. Serial sections for histologic study were made from the thyroid of 80 embryos. Of these, 47 were from hens fed the synthetic diet and 33 from hens fed the control ration. The glands were embedded in paraffin and stained routinely with hematoxylin and eosin. In most embryos only the left lobe of the thyroid gland was removed. The right and left lobes were always found to be in the same stage of development. The embryos varied in age from the 12th day through hatching. The age is recorded as that at the end of a particular day of incubation.

OBSERVATIONS

The vitamin B-12 content of eggs from hens on the complete practical-type ration ranged from approximately 4.0 to 15 μg. per gram of yolk. The vitamin B-12 content of the egg from hens fed the synthetic-type diet, without supplemental vitamin B-12, ranged from 0.2 to 4.0 micrograms per gram of yolk. The size of the thyroid gland after the 12th day of incubation is given in Table 1. Twenty-two (74%) of thirty glands from B-12 defi-

Received May 2, 1956.

[1] This work was supported in part by grants-in-aid from the U. S. Public Health Service, National Institutes of Health, Bethesda, Md., Lederle Laboratories, Pearl River, N. Y., and Commercial Solvents Corporation, Terra Haute, Ind. Inositol was obtained through the courtesy of Corn Products Refining Company, Argo, Ill. Folic acid was supplied by Lederle Laboratories, biotin by Hoffman-La Roche, Inc., Nutley, N. J. and the rest of the B-vitamins by Merck & Co., Rahway, N. J. Soybean oil was supplied by the Buckeye Cotton Oil Company, Cincinnati.

TABLE 1. SIZE OF THYROID GLANDS REMOVED FROM 59 EMBRYOS

Age in days	Normal chick (mm.)†	B-12 deficient embryos (mm.)†
12	1.0×1.0 0.9×1.0	1.0×1.0 1.0×1.0
13	1.0×1.3 1.0×1.3	1.2×1.2 1.2×1.5
14	1.2×1.5 1.0×1.3	1.2×2.8 1.2×1.4
15	1.3×1.5 1.3×1.5	1.5×1.5 2.5×3.0
16	1.3×1.6 1.5×2.0 1.6×2.0	1.8×1.9 2.8×3.0
17	1.7×1.96[a]	1.73×2.26[b]
18	1.3×1.9 1.9×2.0	2.5×2.9 3.2×3.6
19	1.6×1.8 1.8×1.8 2.0×2.0	1.8×2.5 3.2×3.6
20	1.6×1.8 1.5×1.9	3.0×3.8 3.3×3.7
21	1.2×1.5[d] 1.4×2.0[d]	2.8×2.8[c] 3.0×3.0[c]
22		3.0×3.5[c] 3.1×3.8[d] 4.0×4.0[d]
24		3.0×3.3[e]

† Each set of figures represents the two largest diameters of the left thyroid gland of the chick. All measurements are from different chicks.

[a] Average of 9 glands.
[b] Average of 8 glands.
[c] Did not hatch.
[d] Hatched.
[e] One day post-hatch.

cient embryos examined from the 12th through the 22nd day had one or both diameters greater than 2.5 mm. Of the 31 thyroids examined from normal embryos, only 5 had a maximum diameter of 2.0 mm. and none were as much as 2.5 mm. On the basis of a P value of less than .001 it may be concluded that the difference between the size of the thyroids of the normal and the B-12 deficient chick embryos is significant. The thyroid gland in two B-12 deficient chicks examined on the first day after hatching was approximately twice the size of that observed in normal chickens of the same age. The thyroid gland in the deficient embryos usually appeared more vascular than those in the controls.

Histologically the thyroid glands from the twelve-day-old B-12 deficient embryos were composed primarily of cords of cells two layers thick.

There were few very small follicles (Fig. 1). The cells in the periphery of the gland were more compact and showed less differentiation into cords than the cells in the center of the glands. The tissue was extremely vascular at this time. The thyroid from the twelve-day normal embryos showed many follicles throughout the gland. Only a few cells near the periphery had not as yet formed follicles.

The thyroids from the B-12 deficient chicks from the 13th (Fig. 2), 14th (Fig. 3), 15th, 16th and 17th day (Fig. 4) showed a progressive increase in the number of follicles. The deficient thyroids when compared to the nor-

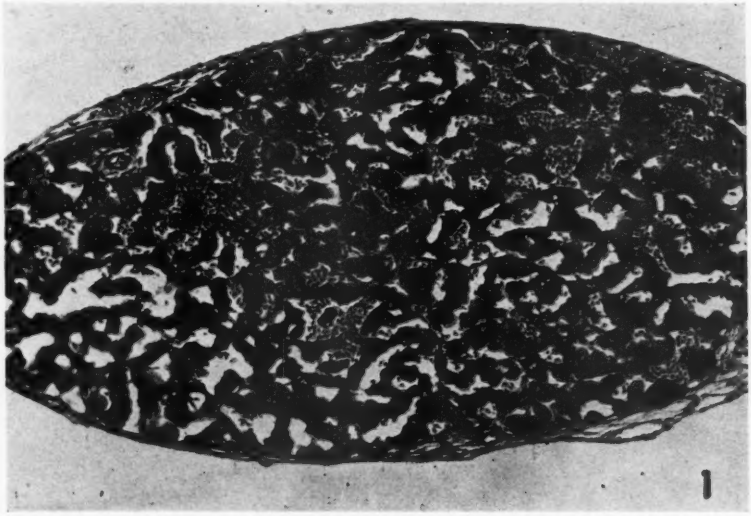

FIG. 1. Embryo 107-28. Vitamin B-12 deficient ration. Twelve days-of-age. The gland is represented by cords of large epithelial cells. Very few follicles have formed. The gland is very vascular. H. &. E. stain ×150.

mal of the same age showed fewer follicles with a greater irregularity in their size and a greater vascularity of the tissue (Fig. 2). The cells lining the follicles of the 16-day embryos were either flat or low cuboidal as compared to the cuboidal epithelium of the normal gland. The development of the thyroid in the deficient embryos always appeared to be approximately 24 to 72 hours behind that in the controls of corresponding age (Figs. 3 and 4).

The normal embryos after the 18th day of incubation showed a complete transformation of the cords of thyroid cells into typical follicles. They varied only a little in size. Cuboidal cells lined these follicles. The lumens were filled with a pink staining colloidal material. In contrast to the normal, the thyroid from the deficient embryos of 20 days showed cords of thyroid cells representing immature follicles (Fig. 5). The epithelium lining these folli-

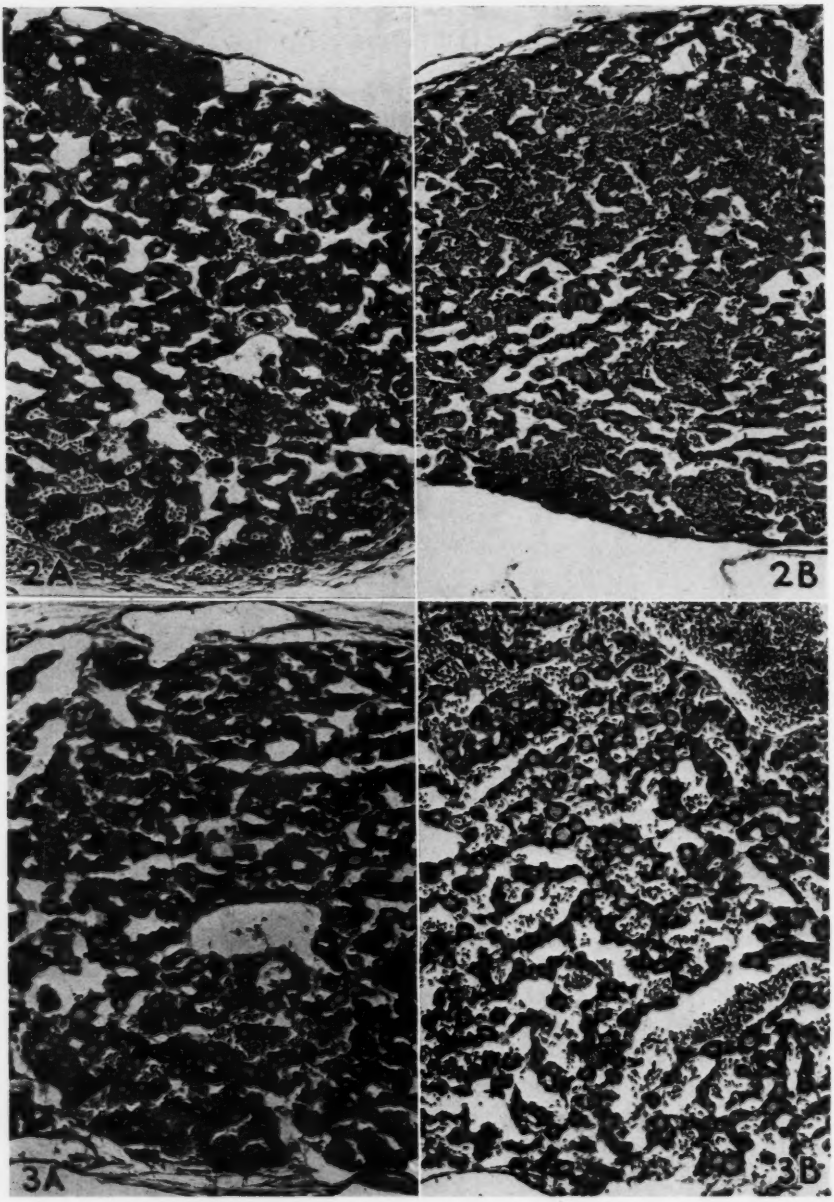

FIG. 2. A. Embryo 89-11. Normal ration. Thirteen days-of-age. There are many small follicles present in the normal gland. B. Embryo 107-25. Vitamin B-12 deficient ration. Thirteen days-of-age. Only an occasional follicle is present in this gland. The variation in development of the normal and the deficient glands is nicely shown in Figures A and B. H. & E. stain ×150.

FIG. 3. A. Embryo 89-7. Normal ration. Fourteen days-of-age. B. Embryo 98-25. Vitamin B-12 deficient ration. Fourteen days-of-age. The thyroid gland is more vascular in the deficient embryo (Figure B) than the normal as shown in Figure A. H. & E. stain ×150.

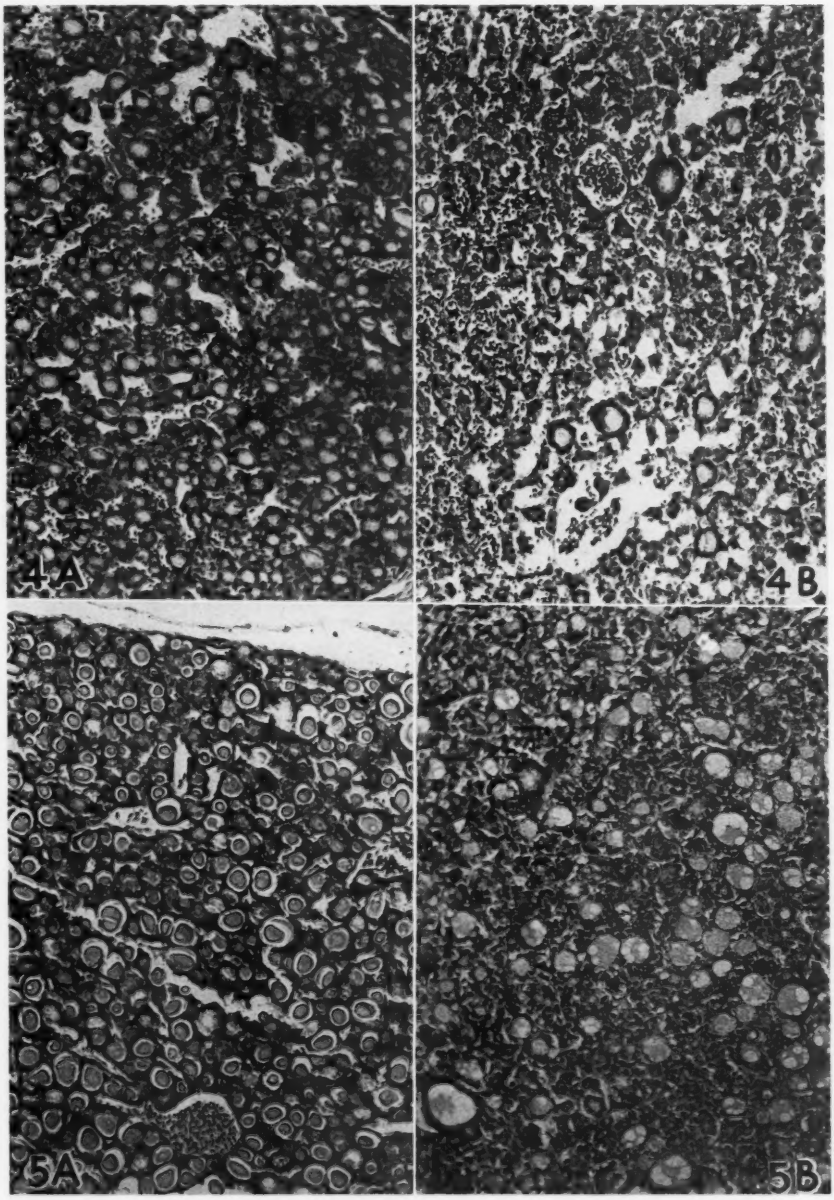

FIG. 4. A. Embryo 108-9. Normal ration. Seventeen days-of-age. B. Embryo 5. Vitamin B-12 deficient ration. Seventeen days-of-age. The variation in the rate of development of the follicles in the normal (Figure A) and the deficient thyroid is nicely illustrated by these two Figures. There is a difference in the number of folicles in differ-

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cles varied in size from cuboidal to a flat type of cell (Fig. 6). Colloid was present in the lumen of the follicles; however, there was a variation in the intensity of its staining. Varying sized vacuoles were present also in this colloid (Fig. 6). Coalescence of follicles occurred in all of the older embryos regardless of the presence or absence of vitamin B-12.

Two B-12 deficient embryos that failed to hatch were removed from the shell on the 21st day of incubation. Numerous follicles that varied in size were present within the thyroid gland. The epithelial cells lining these follicles also varied in size and shape; some were flat while others were either low or normal appearing cuboidal cells. Many of the follicles did not have any colloid, while others showed varying amounts of pink staining material approaching that present in the gland of the normal embryos. The intensity of the staining of the colloid varied in the follicles of the deficient embryo when compared with that of the normal. Many of the follicles had coalesced, while others showed only a narrow zone of pink staining material separating one follicle from another. The thyroid glands from 21-day B-12 deficient embryos usually were completely developed, while the thyroids from normal embryos of 18 days were equally matured.

DISCUSSION

The development of the thyroid gland from vitamin B-12 deficient chick embryos appears to be 24 to 72 hours behind that of the normal chicks. Follicles progressively increase in number and in size after the 10th day of incubation in the normal and the 12th day of incubation in the B-12 deficient embryos. There is a progressive decrease in the vascularity of the thyroid gland accompanying the increase in the number of follicles. The epithelial cells lining the thyroid follicles of the normal embryo are usually cuboidal, while those in the deficient chicks vary between cuboidal and flat epithelial cells. The colloid within the follicles of the deficient embryos varies in its staining reaction. In fact, the colloid is completely absent from many of the follicles in the 21-day-old B-12 deficient embryos. These pathologic changes suggest that the thyroid gland may be hypofunctional in

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ent B-12 deficient embryos. This 17-day-old embryo has fewer follicles than the 14-day-old one shown in Figure 3B. H. & E. stain ×150.

Fig. 5. A. Embryo 103-12. Normal ration. Twenty days-of-age. The follicles in a normal gland vary a little in size. The colloid stains uniformly. The clear area between the colloid and the cuboidal cells apparently represents an artefact. B. Embryo 108-20. Vitamin B-12 deficient ration. Twenty days-of-age. The follicles in a deficient gland vary widely in size. There is considerable variation in the staining of the colloid. Note the vacuolation of the colloid within the different follicles. If the latter should represent only an artefact, then there must be some fundamental difference in the colloid in the normal and the deficient gland to account for these variations. H. & E. stain ×150.

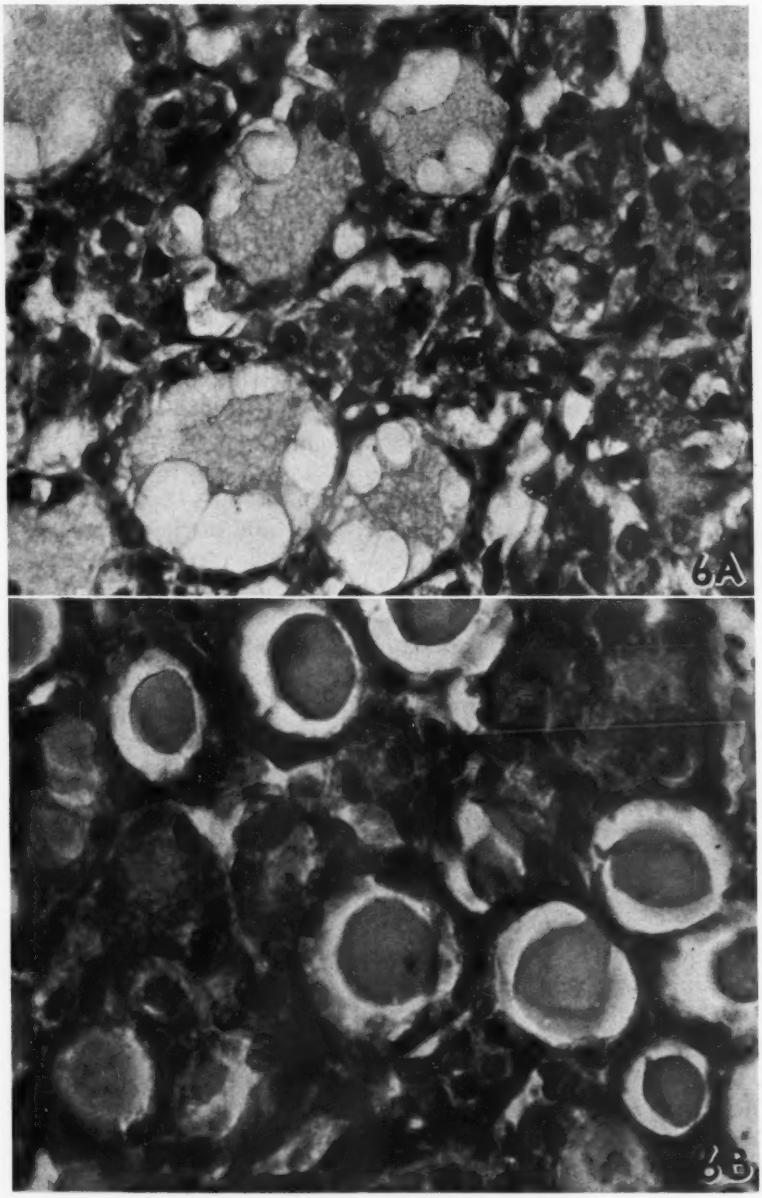

FIG. 6. Same as shown in Figure 5 in a higher magnification. The cells lining the normal follicles are usally cuboidal (Figure A), while many are flat lining the follicles in the deficient embryos (Figure B). The variations in the colloid are nicely shown in these two illustrations. H. &. E. stain ×250.

vitamin B-12 deficient embryos. The size of the gland in deficient embryos is much larger than that of the normal embryos of corresponding age. This increase in size is more clearly shown after the 17th day of incubation (Table 1). It is of interest to note that the peak of embryonic mortality occurs in the 17th day of incubation (3). Any embryos that survive this period would normally be expected to have a higher amount of vitamin B-12 in the egg. It was pointed out previously (2) that vitamin B-12 deficient embryos could be obtained from some hens after a period of only four weeks on the B-12 deficient diet. Other hens required a longer period for depletion. This was interpreted as a factor of the amount of vitamin B-12 stored in the body of the hen. The degree of abnormal characteristics exhibited in embryos from the hens on the low B-12 diet may be due to the varying degrees of storage of the vitamin in the hen.

The significance of the role of the thyroid in the pathogenesis of the lesions previously reported in the viscera of chick embryos (2) is not recognized at this time. It would be of interest to correlate the function of the thyroid of these vitamin B-12 deficient embryos with that of the normal. Hansborough and Khan (5) studied the function of the normal thyroid gland of chick embryos with radio-active iodine. No iodine was found within the gland earlier than on the eleventh day. They noted at this time in the normal embryo the first appearance of "discrete follicles—definite lumina surrounded by cuboidal cells." Iodine was observed by Trunnell and Brayer (6) to concentrate within the thyroid gland on the tenth day of incubation. Due to the different methods of expressing the age of the embryo in these two studies (5, 6) the age of the embryo when iodine is first observed to concentrate in the gland is within experimental error. These experimental observations of Hansborough and Khan (5) and Trunnell and Brayer (6) would suggest that the thyroid glands in the chick begin to function with the development of the follicles. The variation in the time of appearance of thyroid follicles in the chick embryo as shown by different investigators may be attributed to experimental errors, such as the time interval between the laying of the egg and its incubation, the temperature of the eggs during this interval and possibly the variation in the content of vitamin B-12 within the egg.

SUMMARY

The histologic characteristics of the thyroid gland in vitamin B-12 deficient chick embryos are described. The rate of development of the thyroid in the vitamin B-12 deficient embryos is 24 to 72 hours behind that of the normal chick embryo. The gland from the deficient embryo is larger, the follicles vary in size, and the amount of colloid is less than that present in the normal embryo. This study suggests that the thyroid from vitamin B-12 deficient embryos may be hypofunctional.

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THE GONADAL REGULATION OF CHOLESTEREMIA IN THE RAT[1]

LOUIS C. FILLIOS[2]

Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts

ALTHOUGH the subject of many earlier investigations in both humans and experimental animals, the exact role of the gonadal system in the atherogenic process and cholesterol metabolism is obscure (1, 2). Recent work from this laboratory has shown a striking sex associated resistance to hypercholesteremia among rabbits (3). Males were found to have lower serum cholesterol levels than females. Furthermore, males, as a group, also demonstrated a lower cholesteremic response to a discriminating dose of dietary cholesterol. This sex difference among rabbits was shown to be related closely to the production of the gonadal hormones.

Microscopic arterial lesions have been induced in the rat by a variety of techniques (4–8). Rats that had been fed diets containing cholesterol and sodium cholate demonstrated grossly visible atheromatous plaques which, upon microscopic observation, revealed foam cells and intracellular lipid infiltration of both the intima and media (8, 9). Since an intimate association between deranged cholesterol metabolism and atherosclerosis has been extensively documented for several species, interest in this animal for the study of cholesterol metabolism should be enhanced by the above observations.

In the present study, male rats were found to have lower serum cholesterol levels than females. Castration and/or sex hormone treatment of these rats was shown to influence cholesteremia both before and after the introduction of a hypercholesteremic feeding procedure. The regimen adopted for this study is a modification of the atherogenic diet cited above (9).

METHODS

The rats used in these studies were adult albino rats of the Charles River Strain. The animals were kept in individual cages in a temperature-controlled room (72° F) and were weighed weekly. Blood for serum total cholesterol determinations was collected via the tail vein. For terminal studies, the animals were anesthetized with ether and bled by cardiac puncture. The serum total cholesterol determinations were carried out by the method of Abell *et al.* (10).

Received May 25, 1956.

[1] Supported in part by grants-in-aid from the National Heart Institute (No. H-136 C4) of the National Institutes of Health, Public Health, Service Bethesda, Maryland; the Albert and Mary Lasker Foundation, New York; the J. M. Kaplan Fund, New York; and the John H. Hartford Memorial Fund.

[2] A portion of this work was done while the author was a Fellow of the National Heart Institute.

First, the effects of castration and sex hormone administration in the male and female rat (fed only Purina Chow) were studied. The hormone treatments were of 21 days duration. Next, the effects of castration and sex hormone administration on the hypercholestermic response of male and female rats were studied. For this latter experiment the rats were offered a diet which consisted of cholesterol 5%, sodium cholate 1.5%, casein 20%, corn oil 8%, cod liver oil 2%, sucrose 59%, salts 4%, vitamins and trace nutrients as previously reported (11), to each 100 gm. of diet, 6 gm. of Cellu-flour was added. Sixteen gm. of this diet was offered daily to each rat for a period of 28 days. The hormone treatments were begun 21 days before the introduction of the above diet and continued during the 28-day dietary treatment period. At the end of this 49-day study, each rat was anesthetized and then bled by cardiac puncture. Only 1 rat out of 72 in this latter trial was eliminated from consideration because of incomplete food intake.

In all these experimental trials, each rat that received the androgen treatment was injected intramuscularly twice a week with 1.25 mg. of testosterone propionate in 0.05 ml. of sesame oil. Those that received the estrogen treatment were injected intramuscularly twice a week with 0.025 mg. of estradiol dipropionate in 0.05 ml. of sesame oil. The control rats received two intramuscular injections of 0.05 ml. of sesame oil each week.

RESULTS

A. *The Effect of Castration and Sex Hormone Treatment on Cholesteremia in the Rat Fed only Purina Chow (Table 1)*

A sex difference between male and female rats was established.[1] Males, as a group, have lower cholesterol levels than do females ($t = 12.9$; $P < 0.001$). This sex difference is apparently due in part to the production of the gonadal hormones since estradiol treatment elicited a significant increase in the cholesterol level of male rats ($t = 7.9$; $P < 0.001$). Estrogens have been known to promote elevated lipemic states in both rats (12) and birds (13). Loeb (12) found that the administration of estradiol benzoate to male rats (fed a high fat diet) caused an elevation of their serum total lipids and an increased storage of body fat. On the other hand, Rosenman *et al.* (14) found that estradiol had no effect on the serum cholesterol levels of normal rats.

Castration of either male or female rats promoted elevated serum choles-

TABLE 1. THE EFFECT OF SEX HORMONES AND CASTRATION ON CHOLESTEREMIA IN RATS (Mg. of total cholesterol per 100 ml. of serum)*

Hormone treatment (21 days)	Males		Females	
	Intact	Orchidectomized	Intact	Ovariectomized
None	60 ± 0.85† n = 138‡	84 ± 2.80 n = 37	81 ± 1.37 n = 53	100 ± 3.16 n = 17
Testosterone propionate	58 ± 2.67 n = 14	68 ± 1.85 n = 24	88 ± 2.67 n = 14	67 ± 1.60 n = 14
Estradiol dipropionate	83 ± 2.67 n = 14	92 ± 3.45 n = 24	87 ± 4.01 n = 14	115 ± 4.55 n = 14

* Expressed as mg. % in the text.
† Mean ± standard error of the mean.
‡ n = number of individual rats.

terol levels. The *orchidectomized* rats were found to have an average serum total cholesterol value which is significantly higher than that of the intact control males ($t=7.9$; $P<0.001$). Similarly, the *ovariectomized* rats also demonstrated a higher average level than the intact females ($t=4.9$; $P<0.001$). The serum total cholesterol level of the female castrates appears to have been higher than that of the male castrates. Since both male and female rats were castrated after reaching sexual maturity, residual sex differences in lipid distribution characteristics of the pre-castration state may have been still in effect.

Estradiol treatment of either orchidectomized ($t=3.0$; $P<0.01$) or ovariectomized rats ($t=9.0$; $P<0.001$) accentuated the hypercholesteremia of both these castrates. On the other hand, testosterone treatment caused a marked decrease in the cholesterol levels of both orchidectomized ($t=3.0$; $P<0.01$) and ovariectomized animals ($t=9.0$; $P<0.001$).

B. *The Effect of Castration and Sex Hormone Treatment on the Hypercholesteremic Response of Rats Fed a Diet Containing Cholesterol and Sodium Cholate (Table 2)*

Female rats displayed a greater hypercholesteremic response after 28 days on the "cholesterol-cholate" dietary regimen (see methods) than did the male rats. The six females had a final average serum cholesterol level of 598 mg.%; the males, 222 mg.% ($t=3.6$; $P<0.01$). In support of this observation, Aftergood *et al.* (15) also found a sex difference in cholesteremic response among rats fed diets containing cholesterol.

Estradiol treatment tended to increase the cholesteremic response of male rats fed the "cholesterol-cholate" diet. Recently Moskowitz *et al.* (16) also observed that estrogen treatment increases the serum cholesterol response in rats fed diets containing cholesterol.

When female rats were treated with testosterone, they displayed a sig-

TABLE 2. THE EFFECT OF SEX HORMONES AND CASTRATION ON THE HYPERCHOLESTEREMIC RESPONSE OF RATS FED THE "CHOLESTEROL-CHOLATE" DIET FOR 28 DAYS
(Mg. of total cholesterol per 100 ml. of serum)

Hormone treatment (49 days)*	Males				Females			
	Intact		Orchidectomized		Intact		Ovariectomized	
	Days on diet		Days on diet		Days on diet		Days on diet	
	0	28	0	28	0	28	0	28
None	65±3.67† n=6‡	222±34.7 n=6	99±1.23 n=6	217±24.4 n=6	83±2.12 n=6	598±89.7 n=6	105± 4.24 n=6	330±29.7 n=6
Testosterone Propionate	65±3.26 n=6	271±73.8 n=5	77±3.67 n=6	258±36.7 n=6	83±1.25 n=6	294±13.5 n=6	66± 0.71 n=6	204±10.6 n=6
Estradiol Dipropionate	91±3.67 n=6	410±81.2 n=6	102±2.85 n=6	349±54.6 n=6	100±1.00 n=6	705±77.9 n=6	125±10.0 n=6	452±36.3 n=6

* The hormone treatments were begun 21 days before the introduction of the "cholesterol-cholate" diet and continued during the 28-day dietary treatment period.
† Mean ± standard error of the mean.
‡ n = number of individual rats.

nificantly lower cholesteremic response than did the control females ($t = 3.1$; $P < 0.02$). On the other hand, the females treated with estradiol showed a higher cholesteremic response to this dietary treatment than did the controls; but this latter difference was not significant when subjected to statistical treatment.

The cholesteremic response of orchidectomized rats fed the "cholesterol-cholate" diet did not appear to be any different than that of intact males. However, ovariectomy tended to lower the response of female rats ($t = 2.6$; $P < 0.05$). The partial elimination by castration of the wide variability of the cholesteremic response originally observed among intact male and female rats on this dietary regimen is of interest. The range of hypercholesteremia among *intact* males was from 89 to 347 mg.% and among *intact* female rats it was observed to be between 327 and 1009 mg.%. Among *both* male and female castrates, the range was narrowed considerably—162 to 438 mg.%. Therefore, as previously observed in studies with rabbits (3), castration tended to diminish the variability of cholesteremic response among intact male and female rats fed the hypercholesteremic diet.

Testosterone treatment had no apparent effect on the cholesteremic response of orchidectomized rats fed the "cholesterol-cholate" diet while estradiol elicited a slightly increased hypercholesteremia. However, testosterone treatment appeared to protect female castrates against hypercholesteremia, while estradiol administration promoted a higher cholesteremic response than observed among untreated castrated females ($t = 2.4$; $P < 0.05$).

DISCUSSION

The observations reported herein generally support earlier studies carried out in rabbits (3). It appears that "maleness," represented either by an intact gonadal system or by replacement hormone, favors lower serum cholesterol levels in the rat. The sex difference between male and female rats appears to be due largely to the secretion of testosterone in the male and estradiol in the female. Progesterone treatments have also been carried out in another study (17) and our preliminary findings indicate that progesterone-treated castrates have significantly lower levels than estradiol-treated castrates. This latter observation suggests that the higher cholesterol levels observed in the female rat, as compared to the male rat, are due largely to the secretion of the follicular hormone.

It is known that the distribution of lipid material in the female differs markedly from that of the male (18). In order for the female to fulfill the metabolic needs peculiar to its sex, a greater endogenous synthesis of certain lipid material may be necessary. In contrast, the lipid requirements differ for the male rat (19) and a greater emphasis may be placed on the synthesis of certain proteins for the maintenance of secondary sex charac-

teristics such as larger muscle mass. In support of such a hypothesis we found, on the basis of *in vivo* radioisotope studies, that androgen treatment resulted in a decreased biosynthesis of cholesterol (20), while estrogen treatment resulted in an increased biosynthesis of cholesterol in both the castrated female rat (20) and the castrated male rat (17).

SUMMARY

A sex difference in cholesteremia among adult rats was established. Males were found to have lower serum total cholesterol levels than females. The administration of estradiol caused an elevation of the serum cholesterol levels of male rats. Following castration, the serum cholesterol levels of both male and female rats tended to increase. Sex hormone treatment of either male or female castrates revealed that estradiol favors an increased cholesteremia while testosterone has an opposite effect.

A hypercholesteremic dietary regimen of 28 days duration was developed to study the effect of sex on the cholesteremic response of rats. This diet contained both cholesterol and sodium cholate, and it was found that female rats display a significantly higher cholesteremic response than do females. The wide variability of cholesteremic response seen among intact male and female rats was in large measure diminished following castration. Sex hormone treatment of rats fed this diet revealed that testosterone partially protects the female rat from hypercholesteremia while estradiol favors an increased cholesteremia among male rats. Among both castrated males or females estradiol appeared to cause an increased hypercholesteremia. On the other hand, testosterone treatment favored a lesser response among the castrated female rats.

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THE INFLUENCE OF VITAMIN B_{12} DEFICIENCY ON THE UPTAKE OF I^{131} BY THE THYROID GLAND IN ADULT AND EMBRYONIC CHICKENS

T. M. Ferguson, J. B. Trunnell, B. Dennis, P. Wade, and J. R. Couch

Departments of Poultry Science and Biochemistry and Nutrition, College Station, Texas, and The Section of Experimental Medicine, University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, Texas

THE vitamin B_{12}-deficient chick embryo was first described by Olcese *et al.* (1). Additional gross and pathological observations have been reported by Ferguson *et al.* (2, 3). These studies have shown that embryos obtained from hens fed a B_{12}-deficient diet are small in size, edematous and have general body hemorrhages. Focal areas of necrosis were observed in the liver, brain and spinal cord and there was a marked increase in the fat content of the parenchymatous tissues. An enlarged thyroid gland was also reported. Histologic characteristics of the B_{12}-deficient embryo thyroid have been described (4). The thyroid in the deficient embryo contained follicles which showed considerable variation in size and which contained less colloid than was observed in the normal embryo. An apparent delay in development of 24 to 72 hours was observed in the deficient embryo thyroid when compared with the normal.

Trunnell and Brayer (5) reported the time at which I^{131} collection begins in the normal chick thyroid occurs toward the end of the tenth day of incubation. This is in agreement with earlier work (6). Further studies (7) have shown that the biochemical differentiation of the chick embryo thyroid is a gradual process, similar in duration to morphologic differentiation. The present study deals with the ability of the thyroid gland in the deficient hen, and in embryos from the hens, to take up iodine and form iodinated compounds. It also presents preliminary observations regarding specific histochemical measurements.

MATERIALS AND METHODS

Source of birds

(a) Single Comb White Leghorn chicks were placed in batteries with raised wire floors and fed an all-vegetable protein diet deficient in vitamin B_{12} beginning at one day of age. The diet was composed as follows: 35% soybean oil meal, 61% ground yellow corn, 1½% oyster shell flour, 2% dicalcium phosphate (18% phosphorus), and 0.5% salt. Technical feed grade manganese sulfate was added at the level of 160 mg. per lb. The following vitamins were added on a per lb. basis: 2 mg. riboflavin, 5 mg. D-calcium

Received June 5, 1956.

pantothenate, 12.5 mg. niacin, 0.5 mg. menadione, 200 mg. choline chloride, 4000 I.U. vitamin A, 800 I.C.U. vitamin D_3, and 33 mg. procaine penicillin.

At maturity, the birds were placed in individual laying cages with raised wire floors and fed a synthetic diet deficient in vitamin B_{12}. The diet consisted of 62% sucrose, 24% soybean protein,[1] 3% soybean oil and 11% salts mixture. In addition, the following were added per kg. of diet: 20 mg. thiamin, 6 mg. riboflavin, 15 mg. calcium pantothenate, 100 mg. niacin, 4 mg. pyridoxine, 20 mg. para-aminobenzoic acid, 1000 mg. inositol, 10,000 I.U. vitamin A, 3800 I.C.U. vitamin D_3, 0.5 mg. menadione, 24 mg. d-*alpha*-tocopheryl acetate, 2 mg. folic acid, 0.2 mg. biotin, 2000 mg. choline chloride, 33 mg. procaine penicillin, 7.5 gm. methionine and 4 gm. glycine. The salts mixture supplied the following per kg. of diet: 288 mg. manganese, 30 mg. iodine, 559 mg. magnesium, 4 gm. potassium, 246 mg. iron, 4.29 mg. copper, 6.71 mg. zinc and .11 mg. cobalt. The salts mixture supplied 2.5% calcium, 1% phosphorus and 0.5% salt.

(b) A second group of Single Comb White Leghorn hens, four months of age, was obtained from the Texas A. and M. College Poultry Farm, placed in individual laying cages and fed the synthetic B_{12}-deficient diet described above. The hens were fed the deficient diet for 12 to 15 months when used for the iodine uptake studies. These hens had been reared to maturity under farm conditions and had been fed a practical type diet adequate in respect to known nutrients, and which contained added sources of unidentified growth factors prior to the start of the experiment. The vitamin B_{12} content of the egg yolk from hens fed experimental diets was determined at intervals by the method previously described (8).

(c) Four month old pullets (White Leghorn, New Hampshire cross) used in these studies had been reared on a practical ration comparable to that described in *b* above.

(d) Intraperitoneal injections of 5 microcuries of I^{131} in 1 ml. normal saline were used in all tests with the older birds.

Source of embryo

Hens from *Ia* and *Ib* were artificially inseminated with pooled semen from New Hampshire males. Normal control embryos were obtained as described by Trunnell and Brayer (5). Eggs were collected daily, stored in a cold room at 55°, and set at weekly intervals. Some of the eggs were shipped to the M. D. Anderson Hospital, Houston, Texas for histochemical and I^{131} uptake studies of the embryos.

Treatment of embryos and tissues

Eggs containing embryos were injected in the air sac with 5 microcuries of I^{131}. The thyroids were removed from the embryos 24 hours later and counts made in a well counter (5). Thyroids were removed from the pullets and hens 24 hours after intraperitoneal injections of 5 microcuries of I^{131} and counts made as described for embryos. Paraffin sections of thyroids were stained routinely with hematoxylin and eosin. Select sections were stained by the periodic acid-Schiff technique (9). Histochemical tests were made for the presence of sulfhydryl and disulfide according to the method of Barrnett and Seligman (10). Chromatographic analyses of iodinated compounds in the thyroids of both embryos and older birds were made according to the method previously reported (7).

RESULTS

The average I^{131} uptake by the thyroid glands of chick embryos, obtained from hens fed a synthetic-type diet supplemented with vitamin

[1] Drackett C-1 assay protein.

B_{12} was greater than the I^{131} uptake by the thyroids of embryos obtained from hens fed the same diet without supplemental B_{12} (Table 1). In general the amount of I^{131} in the glands increases as the age increases. Variations are noted on the eleventh and twelfth day of the B_{12} deficient group and in both groups after the sixteenth day. The vitamin B_{12} content of egg yolks, determined near the time the above mentioned eggs were incubated, averaged 1.85 μg./gm. The average B_{12} content of normal eggs ranges from 4 to 15 μg./gm. of yolk, based on values determined by this laboratory. In a separate experiment, the I^{131} uptake by the thyroid glands was determined in a 21 day embryo and a day-old chick from hens on different

TABLE 1. I^{131} UPTAKE BY CHICK EMBRYO THYROIDS

Age of embryo (Days)	Average CPM of thyroid	
	Group 1*	Group 2*
	$+B_{12}$	$-B_{12}$
9	8	5
10	21	
11	80	91
12	127	76
13	278	250
14	496	320
15	609	462
16	1407	682
17	833	183
18	1291	1476
19	1590	1772
20	3367	676
21	3892	3513

* Embryos in groups 1 and 2 were obtained from hens fed a synthetic diet which differed only in the presence or absence of vitamin B_{12}.

types of diets. The I^{131} uptake in the case of the 21 day embryo and the day-old chick, obtained from eggs laid by hens maintained on the synthetic B_{12}-deficient diet was only 3% of the injected dose. The uptake of radioiodine by birds of the same age obtained from hens fed a practical type diet containing vitamin B_{12} was approximately 25% of the injected dose.

Chromatographic analyses of the B_{12}-deficient embryonic thyroid glands indicated the presence of iodide, monoiodotyrosine, diiodotyrosine and thyroxine in ratios which were within the normal limits of variation. Periodic acid-Schiff sections did not demonstrate any morphological details that had not been previously reported (4). Histochemical tests made of the thyroid glands of deficient embryos from the twentieth through the twenty-second day of incubation indicated that the sulfhydryl content of both the colloid and the cells was very small, whereas the disulfide content was normal.

Iodine uptake by the thyroids, determined in preliminary studies with three months old pullets reared on a practical type diet deficient in vitamin B_{12}, was approximately 9% of the administered dose. Some of this group of

birds were maintained for an additional month on a similar diet, with added vitamin B_{12}. The I^{131} uptake by this group of birds ranged from 15% to 41% of the dosage.

The I^{131} uptake by the thyroid glands of adult hens which had been maintained on synthetic B_{12}-deficient diet for 12 to 15 months ranged from 0.7% to 2.6% of the administered dose. Intraperitoneal injections of 20 μg. of vitamin B_{12}/hen/day for five days resulted in an I^{131} uptake of 10% of the administered dose in one hen but was not effective in increasing the uptake of two other hens similarly treated.

Chromatograms of the thyroid glands of the pullets and adult hens demonstrated that iodide, monoiodotyrosine, diiodotyrosine and thyroxine were present in approximately normal proportions. The disulfide content of the thyroid glands was apparently normal while only small amounts of sulfhydryl groups were indicated.

DISCUSSION

The general increase in I^{131} uptake by chick embryo thyroids as incubation time increased is in agreement with results previously reported in radioiodine studies with chick embryo thyroids (5, 7). In the latter study (7) some variation was reported in the synthesis of iodinated compounds between the seventeenth and twentieth days. The data presented herein also show some variations between the B_{12}-deficient and B_{12}-supplemented groups (Table 1) during the same period. This is not surprising since the mortality peak for embryos obtained from hens on a B_{12}-deficient diet has been reported to be between the sixteenth and eighteenth day (1). Some of the embryos from this source do hatch, though hatching may be delayed 24 to 48 hours (4). Thus the variations in the latter part of the incubation period in the present experiment is apparently due to variable amounts of vitamin B_{12} in the egg of the developing embryo.

Radioiodine studies (7) showed that the chick embryo thyroid was able to trap iodide prior to ten days of age and that after the appearance of colloid droplets, determined by morphological means, the presence of iodide, monoiodotyrosine, diiodotyrosine, and thyroxine could be detected. Further, these authors reported increasing amounts of these compounds as age increased. Morphological studies of the B_{12}-deficient chick embryo (4) showed a delay of 24 to 72 hours in the development of the thyroid, when compared with normal chicks and that the thyroid gland appeared to be hypofunctional. In the present study, chromatograms showed that the B_{12}-deficient embryo thyroid was able to produce iodide, monoiodotyrosine, diiodotyrosine, and thyroxine. However, the amount of these compounds is apparently less in the B_{12}-deficient group of embryos when compared with the B_{12}-supplemented group. No explanation is readily apparent for the variation between the I^{131} uptake of birds on a synthetic diet and birds on a practical diet. Further studies are being conducted to answer this question.

A reduction in the sulfhydryl content and an apparently normal amount of disulfide was observed by histochemical tests in both the colloid and the cells of the thyroid from B_{12}-deficient embryos, pullets reared on a B_{12}-deficient diet and adult hens fed a diet deficient of vitamin B_{12}. Previous work (11) has shown that vitamin B_{12} is involved in the reduction of disulfide linkages. A decreased level of non-protein sulfhydryl groups, later shown to be due primarily to glutathione, was found in rats fed a B_{12}-deficient diet (12, 13). Work in this laboratory with biochemical methods (14) has demonstrated that the glutathione content in the blood of chicks was reduced in chicks fed a B_{12}-deficient diet.

SUMMARY

1) The thyroid glands found in embryos from B_{12}-deficient hens exhibit reduced ability to concentrate I^{131}. Monoiodotyrosine, diiodotyrosine, thyroxine and iodide are present in the deficient glands as indicated by paper radio-chromatography.

2) The I^{131} uptake of pullets and hens fed a B_{12}-deficient diet may be somewhat increased by administration of vitamin B_{12}.

3) There is marked reduction, bordering upon absence, of sulfhydryl groups in the thyroid glands of vitamin B_{12} deficient embryos and hens.

Acknowledgments

This work was supported in part by a grant-in-aid from the U. S. Public Health Service, National Institutes of Health, Bethesda, Maryland. Inositol was obtained through the courtesy of Corn Products Refining Company, Argo, Illinois. Folic acid was supplied by Lederle Laboratories, Pearl River, New York; biotin by Hoffmann-La Roche, Inc., Nutley, New Jersey; and the rest of the B-vitamins by Merck & Co., Rahway, New Jersey. The *D*-alpha tocopheryl acetate was supplied by Distillation Products Industries, Rochester, New York.

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A STUDY OF THE GLYCOGEN LEVELS OF THE RAT UTERUS AND CERTAIN SKELETAL MUSCLES DURING PREGNANCY[1]

JACK L. KOSTYO

Department of Zoology, Cornell University, Ithaca, New York

STUDIES pertaining to the hormonal control of the glycogen level of the rat uterus have shown that (a) only estrogens are effective in stimulating glycogen deposition (1–3), (b) epinephrine and posterior pituitary extracts rapidly lower uterine glycogen (4), (c) practically all the glycogen is localized in the myometrium (2, 3). This report deals with changes in uterine glycogen during the course of pregnancy, and with changes induced by epinephrine late in pregnancy. These experiments also afforded an opportunity to study glycogen changes in the "stretched" abdominal muscles and the rectus femoris of the leg.

Since the primary objective was to study glycogen changes in the myometrium, the pregnant uterus, emptied of *conceptus*, could not be used. Rich deposits of glycogen in the remaining decidual and endometrial cells (5, 6) would mask glycogen changes in the myometrium. Consequently, the studies were made on the sterile horns of unilaterally pregnant rats.

METHODS

Virgin female rats (180–220 gm.) were employed in these experiments. In order to establish points of reference for comparative purposes, uterine glycogen determinations were made on rats in late proestrus (100% nucleated epithelial cells in the vaginal smear) and in diestrus (48 hours after proestrus). Unilaterally pregnant rats were prepared by ligating the left oviduct on the morning following insemination. Glycogen determinations were made 8–9, 15–16, and 19–21 days after insemination.

In some experiments, the fetuses were removed from the pregnant horn, allowing the placentae to remain and develop (7). On the 13th day of pregnancy the rats were anesthetized and small incisions made in the uterus above each fetus. With slight pressure the fetuses were expressed without disturbing the placentae, and the incisions were sutured with nylon thread.

Pseudopregnant rats were prepared by faradic stimulation of the cervix of females in late proestrus. In some cases, deciduomata were produced by placing three loops of thread in the left uterine horn, 96 hours after stimulation. All pseudopregnant rats were autopsied 9 days after cervical simulation. Whenever spayed females were used, 6-9 days elapsed after the operation before hormones were given.

Estradiol benzoate[2] (0.5 μg. dose) was given 48 hours before autopsy; progesterone[2]

Received June 13, 1956.

[1] Aided by a grant from the Muscular Dystrophy Associations of America, Inc., and in part by the Sarah Manning Sage and Solon P. Sackett Research Funds of the Department of Zoology of Cornell University.

[2] Estradiol benzoate and progesterone were generously donated by the Schering Corporation, Bloomfield, New Jersey.

(2.5 mg. doses) was given 48 hours and 24 hours before autopsy. Epinephrine hydrochloride (Parke, Davis Co.) was injected intraperitoneally in doses of 25 μg. per 100 grams of body weight one hour before autopsy. This dose was previously found to be the minimal amount of epinephrine that could significantly lower uterine glycogen in spayed rats treated with the above dose of estrogen.

All animals were starved 24 hours prior to autopsy primarily for the study on the skeletal muscles, since fasting does not influence uterine glycogen (3). Under Nembutal anesthesia, the *rectus femoris* muscle, a section of muscle from the left side of the abdomen and the uterus were removed, trimmed of extraneous tissue and weighed rapidly on a torsion balance. In pregnant and pseudopregnant animals only the sterile or right horn of the uterus was taken. The methods of digestion and precipitation of glycogen from the tissues were those previously described by Leonard (8) and Walaas (3). The anthrone method was used to determine the glycogen (9) and the latter is reported as mg. of glucose per 100 gm. of tissue wet weight. The data on uterine glycogen is reported in terms of total uterine weight.

RESULTS AND DISCUSSION

Effect of pregnancy: The glycogen levels of uteri from rats in proestrus and diestrus, establishing reference points, are given in Table 1. The results show that the uterine glycogen level of rats in heat was twice that found in diestrus, confirming the work of Boettiger (1). In the 8–9 day pregnant rat, uterine glycogen remained at the level found in diestrus animals (Table 1). At the 15–16th day, there was a small but significant increase in glycogen, and by the 19–21st day the level had reached that observed in proestrus rats. Histochemical examination using the periodic acid leuco-fuchsin technique (10) revealed that this increase in glycogen occurred mainly in the myometrium. Similar results have been described for the cyclic rat by Bo and Atkinson (2). When the fetuses were removed and the placentae left *in situ*, the level of glycogen in the non-pregnant horn at 19–21 days was similar to that observed when the fetuses were present. Rats made psueodpregnant had levels of uterine glycogen similar to those in diestrus rats, and the presence of deciduomata in the opposite horn did not alter the result (Table 1).

TABLE 1. INFLUENCE OF PREGNANCY ON THE GLYCOGEN OF THE UTERUS AND SKELETAL MUSCLE

Experimental conditions	No. of animals	Glycogen (mg./100 gm. of tissue)*		
		Uterus	Rectus femoris	Abdominals
Normal, diestrus	10	102 ± 5	354 ± 12	454 ± 16
Normal, late proestrus	11	*250 ± 11*	*448 ± 17*	*519 ± 18*
Pregnant, 8–9 days	10	113 ± 11	390 ± 21	475 ± 23
Pregnant, 15–16 days	8	*134 ± 10*	382 ± 7	351 ± 33
Pregnant, 19–21 days	12	*216 ± 12*	*428 ± 18*	*309 ± 29*
Pregnant, 19–21 no fetuses	8	*255 ± 16*	*431 ± 20*	*512 ± 27*
Pseudopregnant	4	67 ± 14	426 ± 46	428 ± 6
Pseudopregnant + deciduomata	5	80 ± 10	361 ± 12	373 ± 12

* All average values are presented with the standard error of the mean. Numbers in italics are significantly different from the normal diestrus rat, $P < 0.02$.

These results indicate that beginning on the 15–16th day of gestation there was a gradual increase in the glycogen content of the sterile horn of the pregnant rat uterus, but the level attained did not exceed that observed for cyclic rats in heat. Since estrogen is the only hormone known to increase uterine glycogen (3), it must be assumed that this hormone increases in the rat during pregnancy. Considerable evidence for the placental secretion of estrogens in many species is found in the literature, but early attempts at finding estrogen in the rat placenta were unsuccessful (11–13). The rat placenta, however, must be instrumental in the production of this hormone, because it was only after formation of the placenta that the estrogenic effect appeared. Removal of the fetus but not the placenta produced similar results. Selye and McKeown (5) also suggested an increase in estrogen secretion during the second half of pregnancy, based on the observation that during this period deciduomata could not be produced in the sterile horn of unilaterally pregnant rats. The increase in uterine glycogen during the latter half of pregnancy offers further evidence for increased estrogen secretion. Whether the increased estrogen secretion in late pregnancy comes from the ovaries or the placenta itself remains to be determined.

Concomitant studies on skeletal muscles (rectus femoris and abdominals) indicated that the concentration of glycogen was higher in proestrus than in diestrus, particularly in the leg muscle. Previous experiments demonstrated that relatively large doses of estradiol would elevate skeletal muscle glycogen in spayed rats (14), but it appears that such changes can also occur in the normal estrus cycle. This increase in skeletal muscle glycogen under estrogen treatment is believed to be due to increased secretion of adrenal cortical hormones, and not necessarily to a direct action of estrogen (*ibid.*). When the glycogen levels of the skeletal muscles of diestrus rats were used as points of reference, a significant increase in leg muscle glycogen could be demonstrated at the 19–21st day. No change in the glycogen levels of these muscles occurred as a result of pseudopregnancy either with or without deciduomata present.

The abdominal muscles, however, reacted in a different manner. During the first 8–9 days of gestation, the glycogen level did not change appreciably from that of the diestrus control, but as pregnancy progressed it began to decrease. At the 19–21st day, the glycogen level was significantly lower than in the diestrus control. This drop was attributed to changes resulting from the "stretching" of the abdominal muscles brought about by the increased mass in the abdominal cavity. Support for this opinion was obtained by removing the fetuses and permitting growth of the placentae to continue. Abdominal muscle glycogen failed to decrease in this situation, and a level was obtained similar to that of rats in heat. Furthermore, inspection of the data on individual rats indicated an approximate inverse relationship between the number of young *in utero* and the level of abdominal muscle glycogen. Thus mechanical factors influence the level of

abdominal muscle glycogen during pregnancy and tend to mask the hormonal effect which would be expected to occur.

Effect of epinephrine: Although epinephrine lowered uterine glycogen in estrogen treated spayed rats (4), the different hormonal situation of pregnancy could possibly have altered the effect. A minimal effective dose of epinephrine, however, lowered the glycogen level of the uterus of the 19–21 day pregnant rat, and decreased the level in the leg and abdominal muscles (Table 2).

To further test the possibility that progesterone might modify the glycogenolytic action of epinephrine, a combination of estrogen and proges-

TABLE 2. EFFECT OF EPINEPHRINE ON THE MUSCLE GLYCOGEN OF PREGNANT AND HORMONALLY TREATED RATS

Experimental conditions	No. of animals	Glycogen (mg./100 gm. of tissue)*		
		Uterus	Rectus femoris	Abdominals
Pregnant, 19–21 days	12	216 ± 12	428 ± 18	309 ± 29
Pregnant + Epineph.	6	*77 ± 20*	*204 ± 21*	*82 ± 22*
Spayed	14	78 ± 3	364 ± 13	406 ± 18
Spayed + Estradiol	7	216 ± 19	400 ± 27	
Spayed + Estradiol + Epineph.	5	*106 ± 8*	*280 ± 21*	
Spayed, Progest.	4	83 ± 11	402 ± 9	540 ± 34
Spayed, Progest. + Estradiol	7	174 ± 9	457 ± 31	502 ± 40
Spayed, Progest. + Estradiol + Epineph.	8	*60 ± 7*	*220 ± 24*	*140 ± 27*

* All average values are presented with the standard error of the mean. Numbers in italics are significantly different from their controls, $P < 0.02$.

terone treatment was employed. Spayed rats were given subcutaneous injections of estradiol which would just significantly elevate the glycogen of the uterus. It is interesting to note that this amount of estradiol failed to raise the glycogen level of the leg muscle. Progesterone alone failed to increase uterine glycogen, confirming previous reports (1–3). When both progesterone and estrogen were injected the uterine glycogen level was similar to that obtained with estrogen alone (2, 15). Intraperitoneal epinephrine injections given to rats previously treated with either estradiol or estradiol plus progesterone resulted in a significant decrease in uterine glycogen from control levels. Thus, neither pregnancy nor progesterone appeared to have any effect on epinephrine induced glycogenolysis in the uterus and skeletal muscles.

SUMMARY

Studies on the glycogen level of the sterile horn of the rat uterus during pregnancy showed that an increase in glycogen occurred from the 15th through the 21st day of gestation, approaching a level found for rats in heat. The glycogen content of the uteri of rats on the 8–9th day of pregnancy or pseudopregnancy (with or without deciduomata) was similar to that observed in diestrus.

Epinephrine-induced glycogenolysis was demonstrated in the sterile uterine horns of 19–21 day pregnant rats as readily as in estrogen treated spayed rats. Progesterone given to estrogen-treated rats did not prevent the usual glycogenolytic effect of epinephrine on the uterus.

Concomitant observations on glycogen concentrations in the *rectus femoris* and abdominal muscles revealed a higher level of glycogen during proestrus than in diestrus. During pregnancy an increase in leg muscle glycogen occurred by the 19–21st day of gestation reaching a level similar to that in proestrus rats. Abdominal muscle glycogen, however, decreased during pregnancy and the drop is correlated with the distention produced by the developing fetuses.

Acknowledgment

The author wishes to express his gratitude to Dr. S. L. Leonard for encouragement and counsel during this investigation.

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STUDIES ON THE NATURE OF THE THYROID-PITUITARY INTERRELATIONSHIP[1,2]

R. C. GOLDBERG,[3] J. WOLFF AND R. O. GREEP

Biological Laboratory, Harvard School of Dental Medicine, Boston, Massachusetts, and the National Institutes of Health, Bethesda, Maryland

IT HAS generally been held that hormone secretion by the thyroid and thyrotrophin (TSH) output by the anterior pituitary are homeostatically associated in a "feed-back" type relation. Thus, Hoskins (1) has stated: "When the titer of circulating thyroxine rises, the anterior pituitary is selectively inhibited and the discharge of thyrotrophin is thereby decreased. Contrariwise, episodic or persistent thyroxine deficiency, if sufficient in degree, results in augmented thyrotrophin production with resulting tendency for the production of more thyroid hormone."

Most available data on thyroid activity and pituitary TSH output have been consistent with this view; however, evidence from a number of recent studies has indicated that the actual relationship is perhaps considerably more complicated. The low protein-bound iodine (PBI) of nephrosis (2), the high PBI of normal pregnancy (3) and following estrogen administration (4); and the low PBI found following administration of 2,4-dinitrophenol (DNP) to rats (5) and man (6) have not been clearly linked to inverse changes in the activation of the thyroid by TSH. However, the PBI (or butanol extractable iodine) may not accurately reflect the level of the controlling factor. Changes in the thyroxine-binding protein (e.g. in pregnancy (7)) may alter the concentration of "free" thyroxine in the blood. Moreover, although thyroxine is the major constituent of the PBI, iodinated thyroxine derivatives with different turnover rates and different activities per mole of iodine may appear in the circulation (8, 9) to alter the meaning of the PBI.

Nevertheless, recent studies on the mechanism of depression of PBI by DNP (10) revealed that the TSH effect was depressed by this drug (i.e. decreased iodide concentrating ability and rate of release of iodine from the gland). This paradoxical situation of simultaneously depressed PBI levels and thyroid status is not in harmony with the classical "feedback" hypothesis and constitutes the basis for the present investigation.

Received June 25, 1956.

[1] This study was supported by a grant from the U. S. Public Health Service (A-365).

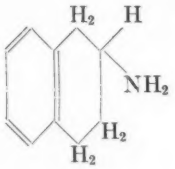
[2] A preliminary report (R.C.G.) of some of the present material has appeared in *Fed. Proc.* **13**: 56. 1954.

[3] Present Address: Department of Medicine, Veterans Administration Hospital, Boston, Massachusetts.

EXPERIMENTAL

Male Sprague-Dawley rats were used in all but one experiment. Propylthiouracil (PTU) was added to the basic diet at a level of 0.1%, and recrystallized 2,4-dinitrophenol (DNP) was added at a 0.2% level, unless otherwise specified. Several attempts were made to use another nitrophenol as a control substance. 2,5-dinitrophenol seemed ideal since it had been reported to be free of calorigenic activity (11). However, several commercial preparations, as well as one synthesized for this study,[4] showed slight activity. This may have been due to contamination by the 2,4-isomer since all samples tested chromatographically showed this impurity. Because of this difficulty, no further controls with nitrophenols were run.

Thyroid iodine (I^{127}) and protein-bound iodine were determined by the method of Taurog and Chaikoff (12). Thyroid-serum iodide concentration (T/S) ratios were determined as described elsewhere (10). Determination of the rate of release of thyroidal iodine (I^{131}) was carried out according to Wolff (13). The QO_2 of liver slices was determined in Krebs-phosphate buffer. Body temperature measurement was carried out with



a two unit iron-constantan thermopile which was inserted 2½ cm. into the distal colon. This unit had a sensitivity of 107 microvolts per degree Centigrade (with the cold junction at 0° Centigrade).

For histological purposes thyroids were fixed in 10% formalin, embedded in paraffin, sectioned at 5 microns, and stained with hematoxylin and eosin. Pituitary glands were fixed in sublimate-formol and 2 micron (paraffin) sections were stained with Goldberg and Chaikoff's (14) modification of Martins' method, the aldehyde-fuchsin stain of Gomori (15), and with the periodic acid Schiff technique. Thyroid histology is graded in Tables 1, 2 and 6 as follows: + = normal, cuboidal epithelium; ++ = columnar epithelium with increased vacuolation; +++ = hypertrophy and hyperplasia with decreased colloid; ++++ = maximal hyperplasia, colloid absent; − = hypoactive, resembling hypophysectomy thyroids.

Plasma levels of DNP were measured according to Parker (16) except that 4-methyl-2-pentanone was used for three successive extractions. This yielded better separation from the colored metabolites of DNP than did ethyl methyl ketone. Levels thus obtained by reading at 430 mμ against plasma of untreated rats did not differ from those obtained by ethanol extraction, suggesting that the reduction products 2-amino-4-nitrophenol and 4-amino-2-nitrophenol were rapidly removed from the circulation.

A nitrogen-free polysaccharide extract (Piromen) of *Pseudomonas*[5] (17) was used as a pyrogenic agent in rabbits. In preliminary experiments it was found that rats did not respond with a febrile reaction in doses up to 1 mg. of this product; some, indeed, suffered a fall in body temperature. The rabbits were injected in the marginal vein of the ear with 50 μg. (5 ml.) every six hours; the controls received a similar amount of

[4] We are indebted to Dr. B. Landau of the Biochemistry Department for this material.

[5] Lot CL-14, generously contributed through the courtesy of Dr. W. F. Windle, Baxter Laboratories, Morton Grove, Illinois.

normal lactate with 1/10,000 merthiolate.[6] Blood for analysis was drawn 26 hours after beginning the treatment.

1,2,3,4-tetrahydro-β-naphthylamine was synthesized by the method of Bamberger and Muller (18). The material was used as the hydrochloride, 4 or 5 mg. were injected intraperitoneally in a buffered solution every 8 hours for 3–6 days.

RESULTS

A. *Effect of Dinitrophenol in Rats Treated with Antithyroid Agents*

1) *Oxygen Consumption.* Liver slices from rats treated with PTU respired at a rate about 30% below the controls. DNP alone raised the QO_2 to about 20% above the controls. Combined treatment with PTU and DNP led to a QO_2 like that following DNP alone. This finding, as well as the elevation of colonic temperature in hypothyroid rats (Fig. 1), served as

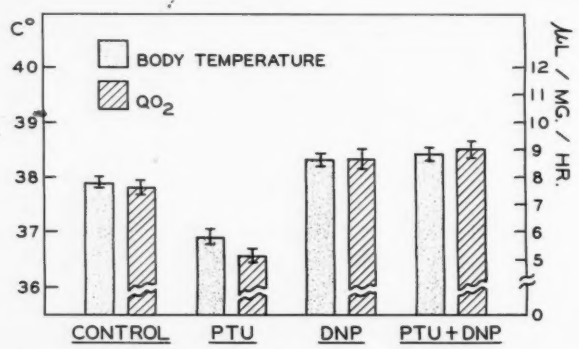


FIG. 1. Colonic temperature and liver QO_2 of rats treated with dinitrophenol and propylthiouracil for 18 days. All diets fed *ad lib.* Control = basic diet, PTU = 0.1% propylthiouracil in diet, DNP = 0.2% 2,4-dinitrophenol in diet. Vertical bars denote standard error of the mean (5 rats/group).

evidence that DNP was metabolically quite active despite hypothyroidism. These results are at variance with those of Alwall (19) and Barker (20), who found almost no metabolic stimulation from nitrophenols in hypothyroid animals, but agree with the observations of Maley and Lardy (21).

2) *Thyroid Morphology.* Clearcut inhibition of goiter formation in propylthiouracil (PTU) treated rats was effected by the administration of dinitrophenol in most of the experiments. While in an earlier experiment (22) and in Experiment I (Table 1), slight or insignificant suppression of goitrogenesis by DNP was observed, striking inhibition of gross thyroid changes were found in Experiments II to VI. An explanation for the variability between experiments or within certain groups has not been found.

Thyroids from rats fed DNP alone were uniformly small and looked

[6] Composition of carrier.

histologically inactive (Fig. 5), whereas control glands revealed a cuboidal epithelium with vacuolated colloid (Fig. 2). PTU-treated rats had large thyroids with tall columnar cells, numerous mitotic figures, non-staining colloid, papillary infolding, and a high degree of vascularity (Fig. 3). In marked contrast were the small pale glands from the rats treated with PTU and DNP. Most of the follicles were large, lined with cuboidal to squamous epithelium with small condensed nuclei, and contained deeply staining colloid (Fig. 4); the peri-acinar capillaries were not dilated as in the PTU-treated rats, i.e., these glands resembled those seen after hypophysectomy or DNP alone. Morphological variation paralleled weight

TABLE 1. EFFECT OF DINITROPHENOL ON RATS TREATED WITH PROPYLTHIOURACIL

Expt.	Treatment	Body weight		Period, days	Thyroid gland		T/S ratio	Histology		Adrenal gland weight, mg./100 gm.
		Initial, gm.	Final, gm.		Weight, mg./100 gm.	Iodine, mg. %		Thyroid	Pit.	
I	PLC (6)†	130–140	298	25	5.3±0.4*					
	0.075 PTU (7)	130–140	230		26.5±4.3					
	PTU+DNP (6)	130–140	223		17.7±5.2					
	0.2 DNP (5)	130–140	230		5.0±0.2					
II	PLC (8)	120–130	278	21	5.3±0.6			++		
	0.1 PTU (8)	120–130	236		29.0±3.2			++++		
	PTU+DNP (5)	120–130	135		7.0±0.6			±		
	0.2 DNP (8)	120–130	141		6.5±0.6			±		
III	PLC (8)	120–130	253	21	6.1±0.6			++	+	
	0.1 PTU (8)	120–130	217		25.6±2.9			++++	+++++	
	PTU+DNP (8)	120–130	134		7.2±1.0			−/++	+/++	
	0.2 DNP (8)	120–130	155		7.2±0.6			−	+	
IV	PLC (6)	130–140	224	17	5.1±0.4	69.0	44±12	++		15.6±0.9*
	0.1 PTU (6)	130–140	179		27.1±5.2	0.43	255±21	++++		14.6±1.3
	PTU+DNP (6)	130–140	142		10.5±5.3	6.4	220±47	−/++		21.2±2.4
	0.2 DNP (6)	130–140	134		6.6±0.3	66.0	16±13	−/+		21.7±2.8
V	PLC (5)	120–130	280	30	5.5±1.2			+	+	
	0.1 PTU (5)	120–130	214		33.3±2.4			++++	+++++	
	PTU+DNP (5)	120–130	146		15.1±6.0			−/+++	+/++	
	0.2 DNP (5)	120–130	148		6.1±1.0			−	−	
VI‡	PLC (6)	200–220	230	30	5.1±0.3					
	0.1 PTU (6)	200–220	210		25.2±3.4					
	PTU+DNP (6)	200–220	208		8.6±4.0					
	0.2 DNP (6)	200–220	205		4.2±0.5					

* Mean± Standard Deviation.
† Figures in parentheses indicate number of rats per group.
‡ Female rats (male rats used in Expts. I–V).
PLC = Purina lab chow; PTU = propylthiouracil: DNP = 2,4-dinitrophenol. Figures indicate % in diet.

variation. The basic reason for the considerable variation in response remains obscure. Rats fed DNP plus PTU ate approximately twice the amount of diet as those receiving PTU alone; it is therefore unlikely that "blocking" levels of the antithyroid drug were not attained.

3) *Pituitary Cytology.* Pituitaries from the rats receiving PTU revealed the characteristic picture reflecting high TSH output, i.e., hyperplasia and hypertrophy of the beta cells with loss of the characteristic granulation, enlargement of the negative image of the Golgi-apparatus, and vacuolation and complete absence of granulated alpha cells (Fig. 6). In marked contrast were the pituitaries from rats receiving DNP in addition to PTU (Fig. 7). Many of these glands revealed none of the signs of beta cell activation noted above. In those rats which showed some thyroid activation

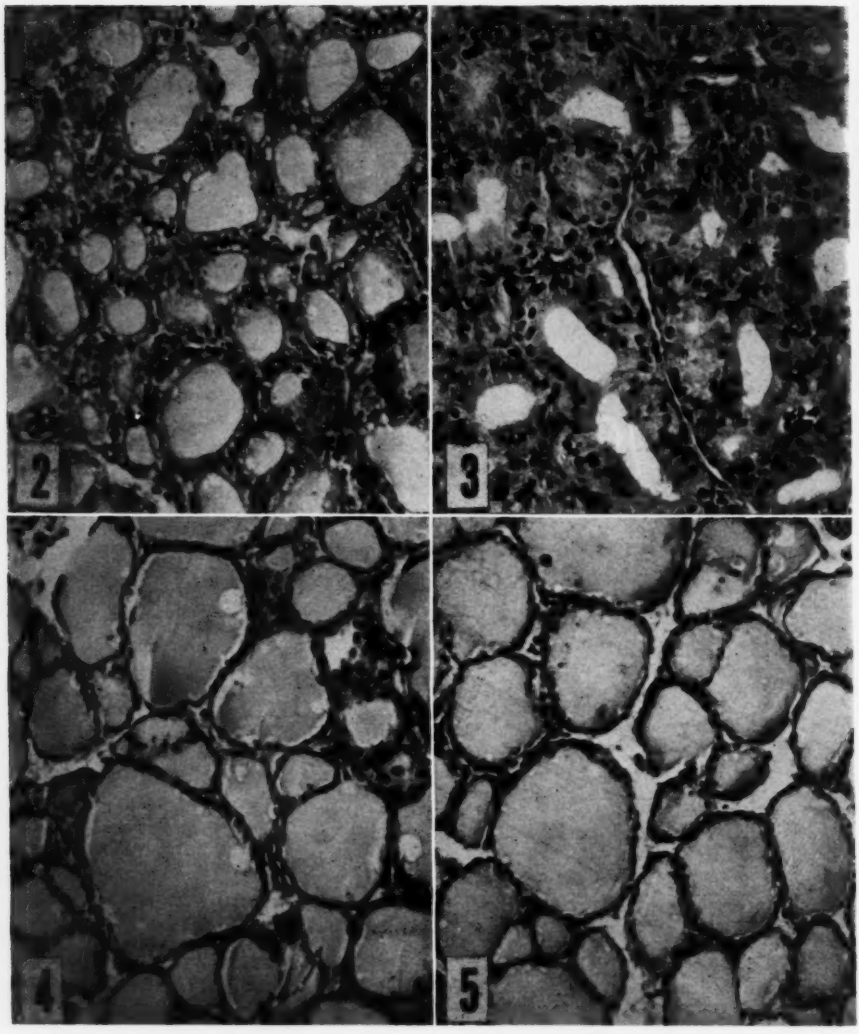


FIG. 2. Thyroid from control rat fed stock (Purina Lab Chow) diet. The epithelium is cuboidal for the most part, the colloid is pale and vacuolated. Hematoxylin and eosin; ×250.

FIG. 3. Thyroid from rat treated with PTU for 21 days. Note the high columnar epithelium, absence of follicular colloid, high degree of vascularity, and papillary infolding. Hematoxylin and eosin; ×250.

FIG. 4. Thyroid from rat treated with PTU and DNP for 21 days. Note the presence of deep staining colloid and squamous epithelium with small condensed nuclei. Hematoxylin and eosin; ×250.

FIG. 5. Thyroid from rat treated with DNP for 21 days. Note that the epithelium is very flat, the follicles are filled with deeply staining colloid; the peri-follicular vessels are difficult to distinguish. Hematoxylin and eosin; ×250.

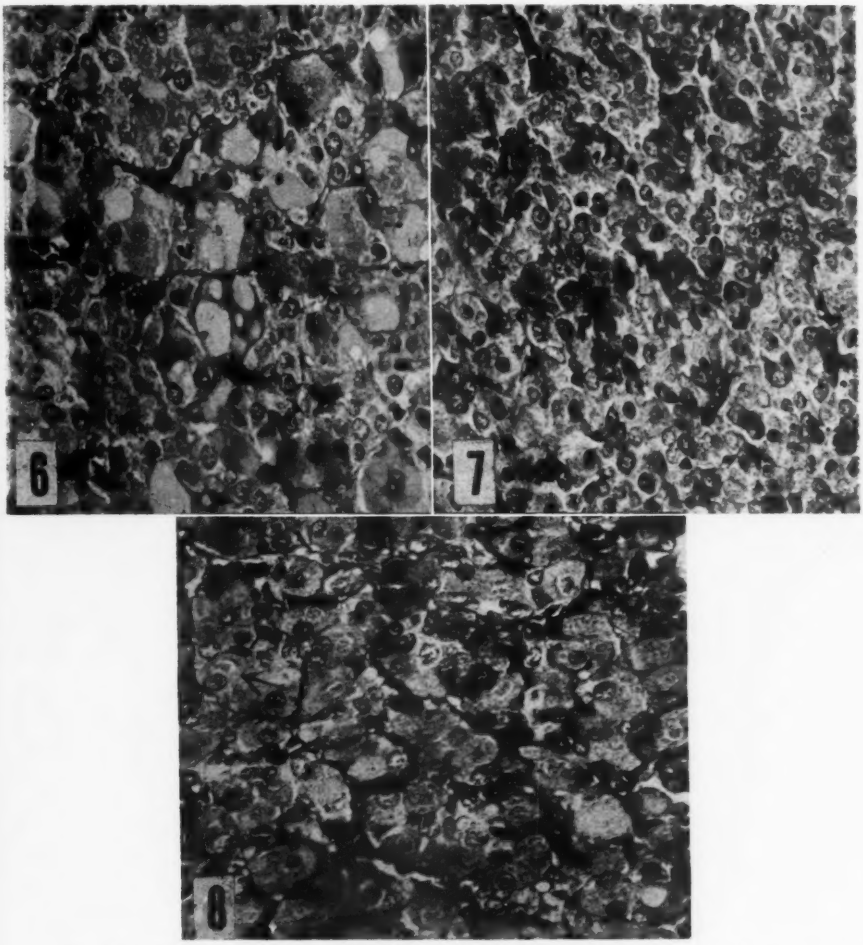


FIG. 6. Anterior pituitary from rat treated 30 days with PTU. There is complete absence of granulated acidophils and numerous hyperplastic, hypertrophied, and vacuolated beta cells can be seen. Martins; ×500.

FIG. 7. Anterior pituitary from rat treated for 30 days with PTU and DNP. The changes noted in Fig. 6 are not apparent here. Acidophils appear dark gray in figure. Martins; ×500.

FIG. 8. Anterior pituitary from castrated male rat, treated with DNP for one month. The marked changes in the delta cells (light in figure) consisting of hyperplasia, hypertrophy, increase in size of Golgi apparatus, and vacuolation are identical to those occurring in castrated rats maintained on the stock diet. Martins; ×500.

there was usually some indication of beta cell hyperactivity, although never of the degree seen in the PTU-treated animals. There was minimal or no degranulation of the acidophils. These cells were numerically and morphologically normal. The pituitaries from the rats receiving DNP alone were essentially normal, although there appeared to be a decrease in the amount of aldehyde-fuchsin positive material in the beta cells; these cells were often quite small and resembled such cells seen in rats treated with thyroxine.

4) *Thyroid Iodine.* The thyroid iodine (I^{127}) content was reduced from 69 mg.% to 0.43 mg.% in the PTU-treated rats; the fall in thyroid iodine of rats treated with both PTU and DNP was not nearly so great (Table 1). Administration of DNP alone had little effect on the iodine content of the thyroid, as has been noted earlier (5).

TABLE 2. ANTIGOITROGENIC ACTIVITY OF DINITROPHENOL IN ADRENALECTOMIZED RATS TREATED WITH PROPYLTHIOURACIL

Treatment	Body weight		Thyroid weight, mg./100 gm.	Thyroid histology
	Initial, gm.	Final, gm.		
PLC (6)#	175 ± 10	165 ± 9*	7.5 ± 1.2*	+
0.1% PTU (7)	175 ± 10	125 ± 12	26.9 ± 5.4	++++
0.1% PTU ± 0.2% DNP (12)	175 ± 10	114 ± 12	8.6 ± 2.8	−/++

Figures in parentheses indicate number of rats per group.
* Mean ± Standard Deviation.
Dietary regimens started 4 days after adrenalectomy. All rats received 2 mg. Compound F acetate s.c. every other day for the duration of the experiment. The rats were sacrificed 14 days after beginning on the diet. PLC = Purina Lab Chow, PTU = propylthiouracil, DNP = 2,4-dinitrophenol.

5) *Thyroid/Serum Iodide Concentration Ratio.* A sensitive index of thyroid activation is the ability of the thyroid gland to maintain a concentration gradient of iodide above plasma or serum (T/S ratio). Whereas DNP significantly inhibited the development of goiter and histological activation, it did not inhibit ($P > 0.5$) the increase in the T/S ratio seen with PTU treatment. The animals fed PTU had ratios averaging 255 and those fed DNP in addition had ratios of 220 as compared to the value of 44 seen in the controls (Table 1). DNP alone effected a questionable reduction in the T/S ratio to 16 ($0.3 > P > 0.2$).

6) *Role of the Adrenals.* A slight increase in the weight of the adrenals occurred in both groups of rats treated with DNP (Table 1). Adrenal corticoids can depress a number of indices of thyroid function, though only when given in large doses (23, and others). To rule out possible participation of the adrenals in thyroid inhibition by DNP, studies were made on bilaterally adrenalectomized rats, maintained on 2 mg. hydrocortisone acetate every other day. As in the intact rats, DNP clearly inhibited the development of goiter and the histological signs of thyroid activation in rats treated with PTU (Table 2); again a few of the rats still showed slight

degrees of TSH stimulation while most approached the level of inactivity (histological) seen in hypophysectomized rats. While the PTU-treated rats (adrenalectomized) lost an average of 50 gm. over the course of the experiments, they still developed thyroid glands four times normal size only 14 days after initiation of the antithyroid regimen. Weight loss, when it occurs, would thus not seem to be a likely explanation for the observed effects.

B. *Effect of DNP on the Anterior Pituitary*

From the foregoing it seemed reasonable to conclude that DNP treatment was followed by signs of decreased thyrotropic activity. A possible explanation for this might be that one of its chemical actions (e.g., uncoupling of oxidative phosphorylation and/or increase in ATP-ase activity) interfered with the synthesis of trophic hormones. The plasma level of DNP as measured in rats two or three days on a diet of 0.2% DNP, was found to be $2.4–5.2 \cdot 10^{-4}$M. While such levels are greater than those usually employed in *in vitro* work, preliminary studies with ultrafiltration indicate that 98 to 99% of DNP added to human plasma is bound to protein at these concentrations.

If beta cell granulation may be presumed to be a sensitive index of pituitary TSH content (and considerable evidence supports such a supposition), the DNP treatment seems to have caused little effect on TSH content in the beta cell. In addition, adrenal enlargement seems to preclude inhibition of ACTH effects. In order to gain additional information on the ability of the anterior pituitary cells to respond to "target organ deficiency" by augmentation in their trophic hormone production, the response of the pituitary gonadotrophes to castration were observed.

Ten adult (200 gm.) male rats were orchiectomized and one week later half of the rats were put on a stock diet containing 0.2% DNP. At sacrifice one month later the DNP-treated rats were found to have gained an average of 16 gm. while the control castrates gained an average of 170 gm. The cytological appearance of the anterior pituitary glands (Fig. 7) was identical in the two groups of rats, i.e., hypertrophy and hyperplasia of the gonadotrophes, vacuolation and formation of the so-called "castration" or "signet-ring" cells, and marked increase in PAS-positive material.

C. *Studies on 1,2,3,4-Tetrahydro-β-Napthylamine (THN) in the Rat*

Ten Cate and Knoppers (24) and others have reported that THN is a potent pyrogenic agent in the rat and administration of THN for 3–6 days produced a rise of about 1.4–1.7 degrees C in the colonic temperature in our experiments. Furthermore, 5 mg. of THN given every eight hours for 6 days resulted in a 25% rise in the QO_2 of liver slices. A 40–50% reduction in the level of PBI accompanied this elevation in QO_2 and body temperature. No consistent effects on the T/S ratio were observed in these experiments (Table 3).

TABLE 3. INFLUENCE OF 1,2,3,4-TETRAHYDRO-BETA-NAPHTHYLAMINE (THN) ON RAT BODY TEMPERATURE, PBI, T/S RATIO, AND QO_2

Experiment	Treatment	Colonic temp., °C	PBI, μg. %	T/S ratio	QO_2 liver μl./mg./hr.
I	Control (6)#	37.6±0.1*	3.3±0.3*	34±7*	
	THN (10)	39.1±0.2	2.0±0.2	26±8	
II	Control (5)	37.5±0.1	3.1±0.2	37±9	6.8±0.4
	THN (7)	38.9±0.1	1.6±0.2	56±10	8.5±0.7

Experiment I: 160 gm. male rats injected with 4 mg. every 8 hours for 3 days.
Experiment II: 200 gm. male rats injected with 5 mg. every 8 hours for 6 days. Sacrificed 6 hours after last injection.
Figures in parentheses indicate number of rats per group.
* Mean ± Standard Deviation.

D. *Studies with a Bacterial Pyrogen*

Fever increases the metabolic rate. In view of the above results with DNP and THN, both of which lead to increased temperature and oxygen consumption, it was deemed fruitful to investigate yet another such agent. The well-known ability of Gram negative bacteria to cause pyrexia led to the choice of Piromen, a nitrogen-free polysaccharide extract from *Pseudomonas*. In Experiment I ten male rabbits were divided into two groups. The *Piromen*-treated rabbits were found to have had sustained temperature elevations of approximately 1.2° C; values depicted in Figure 9 were the determinations made two hours after the last injection. The plasma

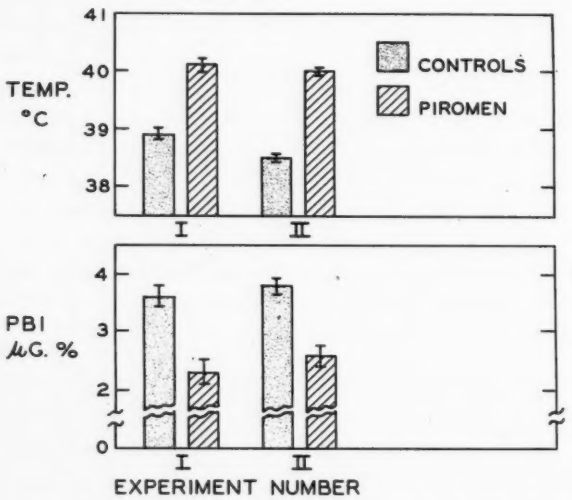


FIG. 9. The effect of a bacterial pyrogen (Piromen) on PBI and colonic temperature in the rabbit. Piromen (50 μg.) injected intravenously every 6 hours for 24 hours. Temperature and PBI determinations were carried out 2 hours after the last injection Five rabbits/group. Vertical bars indicate standard error of the mean.

PBI level was significantly depressed in the *Piromen*-treated animals. In the second experiment the PBI was measured before administration of the pyrogen and blood was again withdrawn for determination after 26 hours (drug administered every 6 hours). The level of PBI was depressed in all of the animals, the average depression being 32%. Chronic administration was not studied because of the refractoriness to fever which develops with repeated injections in rabbits (25).

E. *Studies on the Effect of Ambient Temperature on Thyroid Function in the Rat*

Maintenance of rats at low ambient temperatures has little effect on the level of PBI (26) (Experiment I, Table 4). In sharp contrast is the

TABLE 4. THE EFFECT OF AMBIENT TEMPERATURE ON T/S RATIO, BODY TEMPERATURE, AND PBI IN RATS

Experiment	Ambient temp., ° C	Body weight, gm.	Colonic temp., ° C	PBI μg. %	T/S ratio
I	37.8 (10)#	150–160	39.2 ± 0.2*	1.0 ± 0.2*	
	25.6 (10)	150–160	37.2 ± 0.1	2.8 ± 0.3	
	4.4 (10)	150–160	37.5 ± 0.1	3.2 ± 0.2	
II	37.8 (10)	120–130	39.4 ± 0.2	2.0 ± 0.2	
	25.6 (10)	120–130	37.6 ± 0.1	3.5 ± 0.2	
III	36.7 (5)	130–140	39.2 ± 0.2	1.9 ± 0.2	
	23.3 (5)	130–140	37.4 ± 0.2	3.2 ± 0.3	
IV	37.2 (6)	220–240	39.3 ± 0.2	1.5 ± 0.3	
	25.6 (5)	220–240	37.5 ± 0.1	4.0 ± 0.3	
V	36.7 (6)	150–160	39.2 ± 0.2	1.4 ± 0.2	8 ± 2.7*
	24.5 (6)	150–160	37.5 ± 0.2	3.6 ± 0.3	30 ± 4.3

Figures in parentheses indicate number of rats per group.
* Mean ± Standard Deviation.

significant depression of the PBI found in rats kept at elevated ambient temperatures. In five experiments on male rats of various sizes a fall in the PBI was noted within 24 hours. These animals had free access to drinking water but their hematocrits at time of sacrifice were slightly higher than found in the rats maintained at 23–26° C. This introduces a slight error which, however, would tend to mask a possibly greater actual fall in the level of PBI. Maintenance of rats at such elevated temperatures results in the production of fever (Table 4) averaging 1.7–1.9° C.

The well-known decrease in thyroid activity in rats kept at warm temperatures (27, 28) was clearly reflected in the histologically inactive thyroid epithelium and the decrease in the iodine concentrating capacity (T/S ratio) noted in Experiment V (Table 4). Further evidence of a decrease in thyroid activity at such temperatures was seen in the slowing of the rate

TABLE 5. MEAN BIOLOGICAL HALF-LIFE OF THYROIDAL I^{131}

Experiment	Ambient temp., ° C	$T_{1/2}$+ days
I	26.7 (10)#	1.3
	37.7 (9)	2.0
	4.4 (9)	0.6
II	26.7 (7)	1.4
	37.7 (8)	2.1
	4.4 (7)	0.7

Number of rats per group.
+ Corrected for isotope decay.

of release of thyroidal I^{131} (Table 5). The acceleration of release in rats kept in the *cold* may also be noted in these experiments.

DNP can inhibit goiter formation presumably by interfering with augmentation in thyrotropic output, usually seen after administration of a goitrogen. If such inhibition is related to an increase in O_2 consumption or body temperature, one might expect to find similar inhibition merely by elevating the ambient temperature. Rats fed or injected with antithyroid agents kept at elevated ambient temperatures did not develop goiter (Table 6). Histological examination of the thyroids from these rats revealed slight inhibition of the hypertrophy and hyperplasia found in animals maintained on antithyroid agents at usual temperatures. Additional evidence for incomplete inhibition of accelerated TSH output derives from the thyroid iodine which was depleted after a sojourn in the warm room despite lack of goiter formation (Table 6).

DISCUSSION

2,4-dinitrophenol has significant effects upon the metabolism of the thyroid gland. In addition to its ability to 1) depress the level of PBI (5)

TABLE 6. EFFECT OF AMBIENT TEMPERATURE ON THE THYROID GLAND OF RATS TREATED WITH ANTITHYROID AGENTS

Experiment	Treatment	Ambient temp., ° C	Thyroid weight, mg./100 gm.	Thyroid iodine, mg. %	Thyroid histology
I (18 days)	PLC (8)#	25.6	6.4±0.4*		+
	0.25% MTU† (8)	25.6	19.3±2.1		+++++
	PLC (8)	35.0	5.0±0.2		−
	0.25% MTU (9)	35.0	7.4±0.5		+/++
II (12 days)	PLC (4)	25.6	5.2±0.3*	81	+
	PTU‡ (4)	25.6	13.5±2.2	5	++++
	PLC (3)	36.7	5.6±0.5	64	−
	PTU (5)	36.7	6.3±0.8	12	+/++

# Indicates number of rats per group.
* Mean ±Standard Deviation.
† Methylthiouracil in drinking water. PLC = Purina Lab Chow.
‡ 20 mg. propylthiouracil injected s.c. 2 times a day to insure adequate drug intake despite decreased diet intake in the incubator.

and 2) to inhibit both the concentration and release of I^{131} by the thyroid (10), it inhibits goiter formation and the histological signs of thyroid activity when thyroxine synthesis is blocked by propylthiouracil. A number of explanations suggest themselves to account for the observed facts: 1) that PTU does not act in the presence of DNP; 2) that TSH release is interfered with; 3) that TSH fails to stimulate the thyroid; and 4) that factors other than the hormone level may play a role in the control of thyroid activation.

In view of the fact that PTU was able to depress the thyroid iodine content in DNP-treated rats, it seems unlikely that the antithyroid agent was not active here. The iodine content was, however, not as low as that found in rats treated with PTU alone and this may be the result of the decreased rate of release of thyroid iodine in DNP-treated rats (10).

Direct and non-specific interference with TSH synthesis and/or output by DNP seemed to offer a possible explanation in view of the well-known ability of this agent to interfere with energy yielding or transferring processes. While it was not possible to assess this directly, several observations militate against such an explanation: a) the presence of normally granulated beta cells (presumably the TSH producing site in the pituitary), b) expected responses were obtained by other cells of the anterior pituitary in the presence of DNP as evidenced by 1) adrenal hypertrophy and 2) normal response of the gonadotrophic cells in response to the stimulus of orchiectomy. It is thus unlikely that the TSH inhibiting effects were the result of nonspecific interference with the synthesis of trophic hormones.

Thyroid inactivity in rats treated with DNP and PTU might result from increased "destruction" of TSH even though output of this hormone by the pituitary were greatly augmented. The beta cells of the pituitary of the rat treated with PTU ordinarily undergo a series of characteristic changes that have been correlated with increased TSH output (29). Since these changes were minimal or did not occur in the rats treated with PTU and DNP, it can be inferred that the goiter inhibition was not primarily the result of an enhanced rate of disappearance of TSH from the circulation but resulted from actual reduction of TSH output by the pituitary. In preliminary studies with exogenous (beef) TSH, DNP was *not* able to abolish the increase in T/S ratio produced by small doses of the trophic hormone.

While it is well known that DNP will not duplicate many of the actions of the thyroid hormone (e.g., the repair of myxoedema), it does share several important properties: 1) pyrogenic and metabolic rate stimulating activity, 2) uncoupling of oxidative phosphorylation (30), and 3) ATP-ase stimulating activity (31). If the inhibition of beta cell TSH output was related to the first of these, then other agents which possess these properties might exert a similar inhibitory effect on these cells. Three such (otherwise unrelated) agents (a bacterial pyrogenic extract, 1,2,3,4-tetrahydro-

β-naphthylamine, and elevated ambient temperature) are all similar to DNP in that they lead to a marked fall in the level of PBI together with clearcut evidence of decreased TSH stimulation (histological inactivity, decrease in the iodide concentrating capacity, and decrease in the rate of release of organic iodine to the circulation). Furthermore, the maintenance of rats under such conditions effectively inhibited the development of goiter and the histological signs of thyroid activation in rats treated with antithyroid agents.

While it thus appears not unlikely that the inhibition of TSH release is an effect of various agents or conditions that can mimic the thyroid hormone in certain respects, it is not clear at what point(s) in the homeostatic chain this inhibition is exerted. The beta cells of intra-ocular pituitary transplants in hypophysectomized *rats* are filled with aldehyde-fuchsin positive granules that are not discharged in response to a low level of circulating thyroid hormone (in press, 31a). If these granules are a reliable reflection of TSH, this would suggest that these cells can store TSH but do not release it to the circulation. Such evidence would be consistent with the possibility that the effects of the thyroid hormone are transmitted to the pituitary beta cells via the central nervous system. When such preparations are made in the *rabbit*, however, sufficient TSH is delivered to keep the release of thyroidal I^{131} at the same rate as normal controls (32). Inhibition of TSH secretion has been noted in animals with hypothalamic lesions (33, 34) and in stalk transected animals where reestablishment of the hypophyseal-hypothalamic portal vascular connection has been prevented by a mechanical barrier (35). On the other hand, local injections of thyroxine in large amounts inhibit I^{131} release from the *rabbit* thyroid *only* when the material is placed into the anterior pituitary but not in the hypothalamus (32).

It is impossible at the present time, therefore, to identify the locus of action of the compounds reported here. Suffice it to say that under certain conditions a low level of circulating thyroid hormone (as measured by the PBI) need not stimulate TSH output; moreover, even with essentially complete inhibition of thyroid hormone synthesis by antithyroid agents, TSH output can be blocked by agents which serve to maintain the animal's body temperature and/or metabolic rate at normal or supernormal levels. It thus is quite feasible that not the actual level of circulating thyroid hormone but one or more of its metabolic actions is the important factor in the regulation of pituitary TSH output.

SUMMARY

Dinitrophenol (DNP) prevents the development of goiter and the histological signs of thyroid activation in propylthiouracil (PTU) treated rats with or without intact adrenal glands. DNP alone leads to thyroid atrophy. It has little or no effect in preventing the loss of iodine and does not reverse

the elevation of the thyroid-serum iodide concentration (T/S) ratio usually resulting from PTU treatment.

The cytological changes indicative of agumented TSH output by anterior pituitary beta cells in the PTU-treated rats are inhibited by DNP. Degranulation of the alpha cells is also prevented.

The lack of activity of DNP in the hypothyroid rat reported by others was not confirmed. Studies on QO_2 and body temperature clearly reflect the stimulating effect of DNP in the PTU-treated rat.

DNP does not inhibit the development of castration changes in anterior pituitary delta cells following orchiectomy. This finding, plus the adrenal hypertrophy and the "normal" beta cells of the pituitary in DNP-treated rats suggest that the DNP effect is not a nonspecific inhibition of pituitary metabolism.

Administration of 1,2,3,4-tetrahydro-β-naphthylamine to rats results in: 1) elevation of body temperature; 2) increased QO_2 (liver); and 3) fall in the level of plasma protein bound iodine (PBI).

Administration of a *Pseudomonas* polysaccharide to rabbits resulted in elevation of body temperature and a fall in the level of PBI.

Maintenance of rats at an elevated ambient temperature resulted in elevated body temperature; fall in the level of PBI, a decreased T/S ratio, a decrease in the rate of release of thyroidal organic I^{131}, and inhibition of goiter formation by PTU.

It is felt that the data are not consistent with the simple classical "feedback" theory of thyroid-pituitary interrelations. It is likely that the pituitary cells secreting TSH are influenced not only by the actual circulating level of thyroid hormone(s) but by one or more of its peripheral actions.

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PLACENTAL TRANSFER OF PERCHLORATE AND TRIIODOTHYRONINE IN THE GUINEA PIG

SHOLEM POSTEL

The Medical Services of the Massachusetts General Hospital and the Department of Medicine, Harvard Medical School, 25 Shattuck St., Boston, Massachusetts

THIS report documents (1) production of fetal goiter when perchlorate is administered to pregnant guinea pigs, (2) failure of large doses of triiodothyronine to protect the fetus against perchlorate induced goiter, and (3) studies on the placental transfer of I^{131} labeled triiodothyronine. The findings bear upon the use of these agents in the treatment of thyroid diseases during pregnancy. In addition, they may be relevant to broader aspects of fetal-maternal interrelations.

METHODS

Administration of $KClO_4$ and Triiodothyronine to Pregnant Guinea Pigs

Sixteen guinea pigs in the second or third week of pregnancy were treated as follows. One control group of three pigs received a diet[1] containing 0.48 μg. iodine per gram, distilled water *ad libitum*, and 50 mg. of ascorbic acid orally twice a week. The other pigs were offered a 1% solution of $KClO_4$ instead of distilled water. They were divided into 4 groups which received 0, 8, 16, and 32 μg., respectively, of *l*-3-5-3′-triiodothyronine[2] by subcutaneous injection each day. The solution of triiodothyronine (T-3) was prepared every three days in .05 ml. of 0.1 N NaOH and diluted with 0.9% NaCl. Control animals and those receiving $KClO_4$ alone were given daily injections of saline solution. Initial *ad libitum* consumption of the 1% $KClO_4$ solution was the same as the intake of distilled water in the control group, but after the first week perchlorate intake decreased progressively, particularly in animals receiving no T-3. The over-all mean dose of $KClO_4$ was 152 mg./100 gm. body weight per 24 hours in T-3 injected pigs and 74 mg./100 gm. in the group receiving only $KClO_4$. The mean duration of treatment was 37 days (range 21–48).

One hour after 10–20 microcuries of carrier free NaI^{131} was administered subcutaneously to the mother, the near-term fetuses were removed by hysterotomy under pentobarbital anesthesia. Blood was obtained from the hearts of mothers and fetuses for determination of I^{131} content in a well-type scintillation detector. Fetal and maternal thyroids were excised, weighed, fixed in 5% formaldehyde, and assayed for radioactivity. The counts per minute per gram of thyroid tissue, divided by the counts per minute per ml. of plasma defines the "T/S" ratio.[3]

Received June 25, 1956.

Supported in part by Grant A-849 of the United States Public Health Service.

Presented at the 38th meeting of the Endocrine Society, June 8, 1956, Chicago, Illinois.

[1] Purina rabbit chow.

[2] Smith, Kline and French Laboratories, Philadelphia, Pennsylvania.

[3] "T/S" ratio as defined here differs from the conventional thyroid/plasma ratio for inorganic iodine. Expressing the thyroidal uptake of I^{131} in this way takes into consideration non-uniform distribution of inorganic I^{131} between adult and fetus (1).

Administration of $KClO_4$ to Non-Pregnant Adult Female Guinea Pigs

To determine whether or not goiter could be produced in non-pregnant pigs under the conditions of the experiment described above, 24 young adult virgin sows were divided into two groups, one receiving distilled water, the other 1% $KClO_4$ *ad libitum*. Four controls and four $KClO_4$ treated animals were sacrificed at 30, 60, and 90 days after the beginning of the experiment. "T/S" ratios were determined as described above. In addition, one-half of each gland was analyzed for total iodine content by the method of Barker (1).

Placental Transfer of I^{131} labeled l-3-5-3'-Triiodothyronine

Eighteen studies of the partition of I^{131} labeled l-3-5-3'-triiodothyronine (T-3*) in the maternal and fetal circulations were done. T-3*, prepared by radioiodination of 3-5-diiodothyronine, purified by column chromatography and dissolved in 50% propylene glycol[4] was administered to near term sows one-half hour after an oral dose of 330 mg. of $KClO_4$. At intervals from 5 minutes to 24 hours after the T-3* injection, fetuses were delivered as described above. Appropriate changes of rubber gloves and surgical instruments and use of separate working areas maintained isolation of the fetal and maternal circulations. Fetal and maternal blood samples obtained simultaneously were heparinized and processed as described below. Radioactivity in the thyroid glands of mothers and fetuses was measured to show complete inhibition of iodide trapping. T-3* was injected into a jugular vein exposed under light ether anesthesia in the experiments in which the pigs were sacrificed at one hour or earlier. In all other experiments, i.e., at 4, 12, and 24 hours, it was given subcutaneously. In experiments lasting longer than 4 hours, oral doses of 166 mg. of $KClO_4$ were administered every 6 hours in addition to the initial dose given one-half hour before the T-3* injection. T-3 dosage varied with the specific activity of various lots (15 to 78 millicuries per milligram) and the counting rate demands of the particular experiment. The smallest T-3 dose was 0.3 μg., the largest, 10 μg.

Blood was centrifuged immediately and a small crystal of NaS_2O_3 added to the separated plasma which was acidified to pH 2, and extracted three times with four volumes of thiosulfate saturated n-butyl alcohol. The pooled supernatant was neutralized with NH_4OH and evaporated to dryness. The residue was taken up in 0.1–0.2 ml. of a solution of 3 parts of ethyl alcohol and one of NH_4OH immediately prior to application to Whatman #3 filter paper. Chromatograms were developed in ascending fashion in butanol-dioxane-ammonia and butanol-acetic acid-water. The sites of previously added carriers were identified by coupling with the diazonium salt of sulfanilic acid. When counting rates were favorable, the distribution of radioactivity on the chromatogram was determined using an automatically driven scanning device with an end window detector, a counting rate meter and recorder. Otherwise, as in the case of fetal plasmas, segments of chromatograms were counted in a deep well scintillation counter with a sensitivity of 400,000 counts per microcurie and a background of 15–25 counts per minute. Results are expressed in per cent of administered dose per ml. plasma, corrected to a 1000 gm. body weight. In experiments on the decay of T-3* in newborn guinea pigs, the same analytic techniques were employed. In one experiment, whole fetal carcasses were homogenized. Acidified aliquots were extracted with butanol and chromatographed in the same way as noted above for plasma.

RESULTS

A. *The Effects of Perchlorate with and without Simultaneous Administration of Triiodothyronine*

Feeding 1% perchlorate to pregnant guinea pigs during the last 21 to 48 days of gestation resulted in massive enlargement of the fetal thyroid

[4] Abbott Laboratories, Oak Ridge, Tennessee.

glands. Their over-all mean weight was 491 ± 157.4 mg./100 gm. body weight, compared with control fetal thyroid weights of 32.3 ± 3.44 mg./100 gm. As shown in Figure 1, large goiters developed despite simultaneous administration of triiodothyronine (T-3) in doses up to 32 μg. per day to the mother. The striking perchlorate effect on the fetus is in marked contrast to the unaltered weights of maternal thyroids. This contrast is exemplified in the illustration (Fig. 2) of the *in situ* appearance of typical highly vascular goiters in both fetuses of a one kilogram mother whose excised thyroid gland is included for reference. In this particular case, the

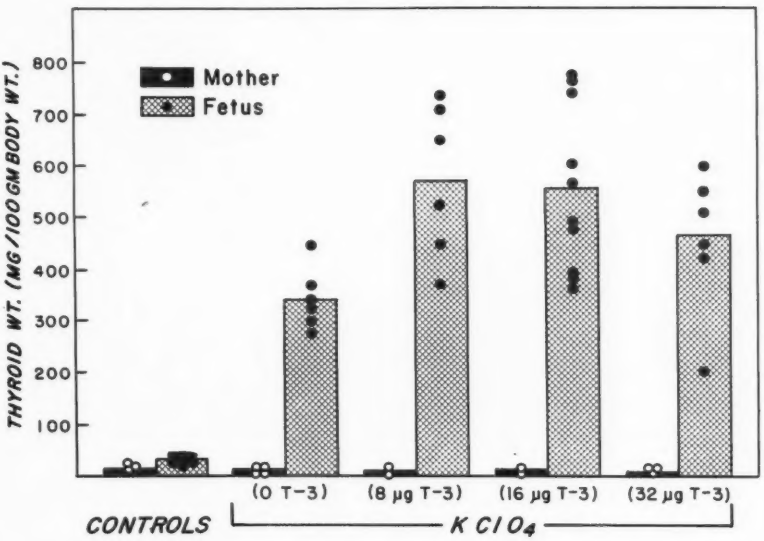

FIG. 1. Thyroid weights (mg./100 gm. body weight) of mothers (black bars) and fetuses (cross-hatched) with and without simultaneous administration of graded doses of triiodothyronine.

sow received 16 μg. T-3 daily. Similarly, microscopic examination showed no changes in maternal glands despite virtual replacement of the normal fetal thyroid architecture by solid cords of cells (Fig. 3). Here, as for the thyroid weights (Fig. 1), it appears that, if anything, goitrogenesis was more intense when triiodothyronine was co-administered.

One hour thyroid to plasma ratios of fetal and maternal I^{131} concentration (Fig. 4) were of interest in providing support of the gross and microscopic impression that the normal fetal thyroid is a relatively hyperplastic gland with a T/S ratio three times that of the adult.

B. *Goiter Production in Non-Pregnant Adult Female Guinea Pigs*

The absence of changes in maternal thyroids after treatment with $KClO_4$ raised the question as to whether or not it is possible, under any conditions,

FIG. 2. *In situ* appearance of thyroid glands of fetuses of 1 kg. mother who received 16 μg. triiodothyronine per day in addition to 1% $KClO_4$. Excised normal sized maternal thryoid is shown above.

to produce perchlorate goiter in adult guinea pigs. Furthermore, the possibility that pregnancy, as such, protects the maternal thyroid had to be considered. One per cent $KClO_4$ solution was therefore fed to non-pregnant adult female guinea pigs. Thyroid weights, histology, "T/S" ratios and iodine content were determined in control and perchlorate treated animals at 30, 60, and 90 day intervals. As shown in Figure 5, there was no evidence, by weight or histology, of perchlorate effects at 30 days, despite definite iodine depletion from 44 ± 22 μg./100 gm. to 13 ± 3.9 μg./100 gm. At 60 days, however, thyroid enlargement and intense hyperplasia were obvious. Inasmuch as the mean duration of exposure of the pregnant sows to 1% $KClO_4$ was only 37 days (range 21–48), it is reasonable to assume that insufficient time had elapsed for goiter to develop in the pregnant pigs.

C. *Partition of I^{131} Labeled Triiodothyronine in Mother and Fetus*

The placental transfer of administered T-3 was tested directly by injection of I^{131} labeled triiodothyronine (T-3*) into pregnant guinea pigs near

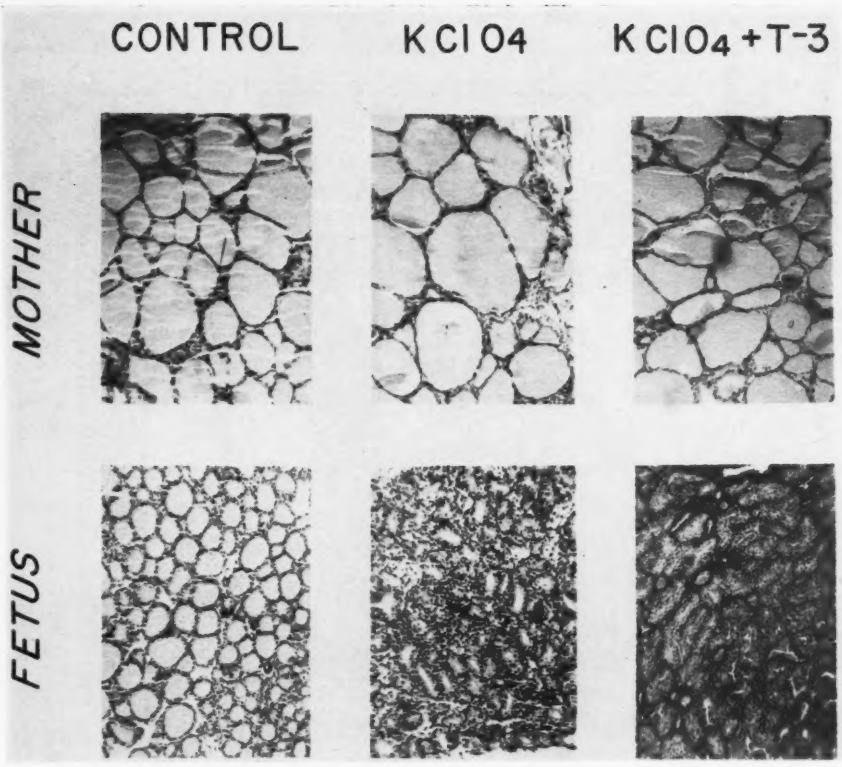

FIG. 3. Microphotographs of thyroid glands of adult pigs and their fetuses with and without daily administration of 32 μg. triiodothyronine.

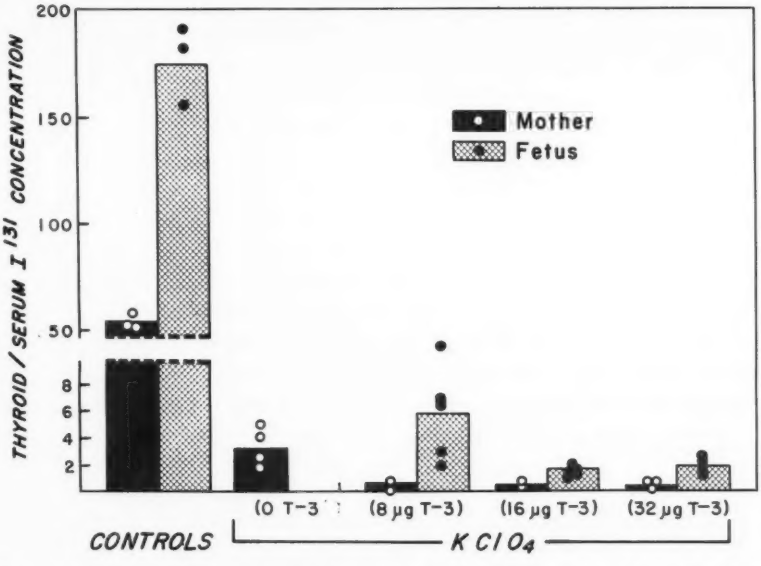

FIG. 4. One hour "T/S" ratios of fetuses (cross-hatched) and mothers (black bars) with and without graded doses of T-3. No blood was obtainable from fetuses of mothers receiving $KClO_4$ alone. Note break in scale.

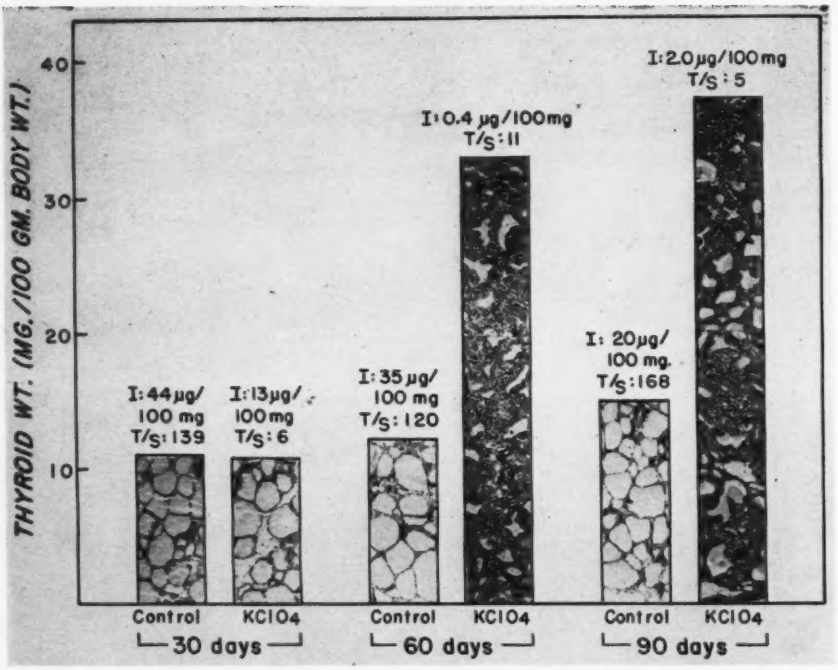

FIG. 5. Mean weights (mg./100 gm. body weight), iodine content, "T/S" ratios and representative histology of adult female guinea pig thyroids after 30, 60, and 90-day periods of treatment with 1% $KClO_4$. Four pigs in each of the six groups.

term. Concentrations of total radioactivity and T-3* in maternal and fetal plasma were determined at intervals up to 24 hours. Figure 6 shows the change in concentration of total radioactivity with time, the lower curve representing fetuses, the upper one the corresponding maternal concentration. In Figure 7 the *ratio* of fetal to maternal concentration is plotted against time. It is evident that the concentration of total I^{131} is not uniform in maternal and fetal plasma until 24 hours have elapsed. Furthermore, delay in equalizing total I^{131} concentration appears to be a function of the rate at which iodide becomes available by degradation of T-3* as indicated in the decay curves (Fig. 8) for chromatographically identified T-3* in the maternal and fetal circulations. These show that fetal and maternal vascular spaces equilibrate at a concentration ratio of 0.05 and that the biological half life of T-3* under these conditions is approximately 10 hours.

D. *The Mechanism of the Concentration Gradient for Labeled Triiodothyronine*

The above noted gradient for concentration of maternally administered T-3* across the placenta could be maintained by any one of the following mechanisms:

1) relative non-permeability of the placenta to T-3*,
2) rapid fetal degradation of freely diffused T-3*,
3) dilution of T-3* by a large quantity of stable T-3 in the fetus,
4) diversion of T-3* into extravascular depots,
5) exceeding the limit of total mass of T-3 which can be transferred across the placenta per unit time even if T-3 freely traverses it.

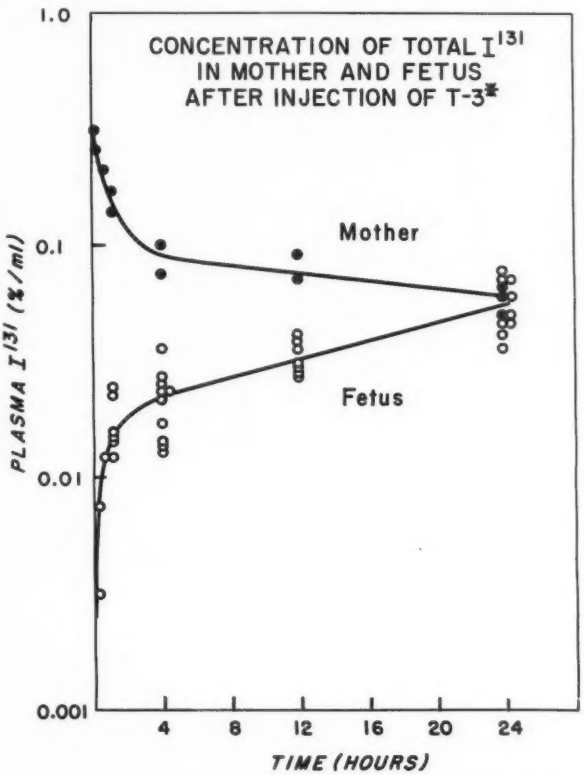

FIG. 6. Change in concentration of total radioactivity in maternal and fetal plasma after administration of I^{131} labeled triiodothyronine to pregnant guinea pigs.

Several experiments relative to consideration of these mechanisms were done. T-3* was administered to pregnant pigs intravenously together with 2.0 mg./kg. of carrier T-3 in one case and 20.0 mg./kg. in the other. The placental gradients for T-3* concentration at one hour were not significantly different (Table 1) from the gradients with microgram doses. This is in keeping with the observation that in all other experiments the fetal/maternal ratios did not correlate with variation in T-3 dose over a range of 0.3 to 10 μg.

The whole carcasses of 4 fetuses were homogenized and assayed for total

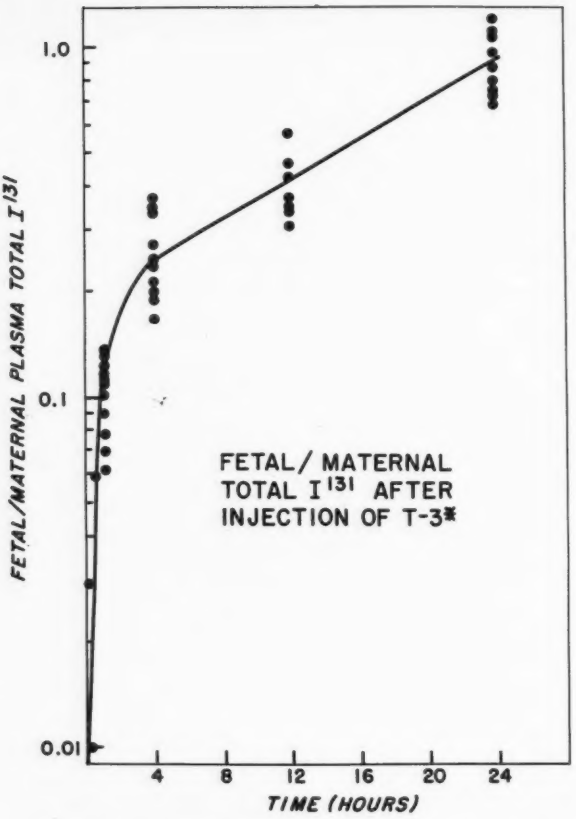

FIG. 7. Ratio of fetal to maternal plasma concentration of total radioactivity after administration of I^{131} labeled triiodothyronine to pregnant guinea pigs.

and T-3* radioactivity one hour after intravenous injection of T-3* into the pregnant sow. As recorded in Table 2, the concentration of T-3* in the body of the fetus was less than that in plasma, on a gram for gram basis

TABLE 1. INFLUENCE OF DOSE OF TRIIODOTHYRONINE ON DISTRIBUTION OF I^{131} LABELED TRIIODOTHYRONINE ONE HOUR AFTER INJECTION

	Dose of T-3 (mg./kg.)					
	0.005		2.0		20.0	
	Total I^{131}	T-3*	Total I^{131}	T-3*	Total I^{131}	T-3*
Ratio of Fetal to Maternal Plasma Concentration	0.13 0.13 0.10 0.11	0.026 0.030 0.023 0.022	0.13 0.16 0.14	0.035 0.072 0.044	0.104 0.113 0.102	0.018 0.032 0.027
Mean	0.12	0.025	0.14	0.050	0.11	0.026

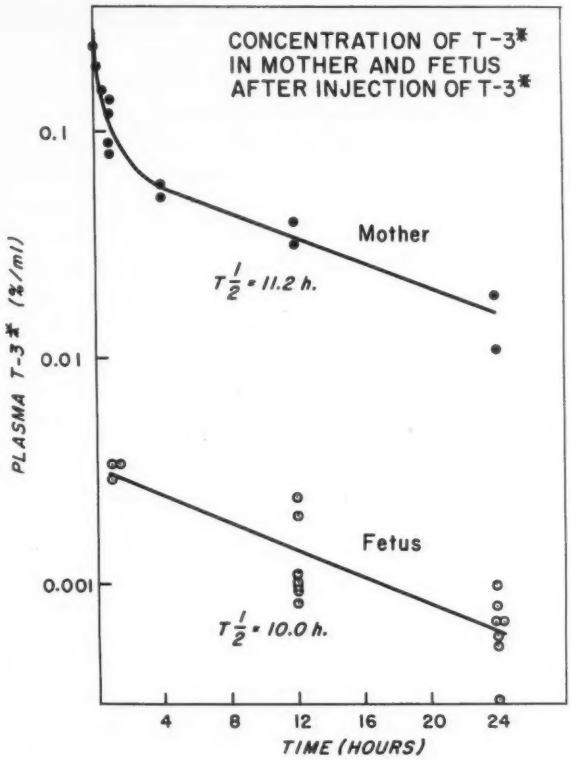

FIG. 8. Concentration of chromatographically identified triiodothyronine in maternal and fetal plasma after administration of I^{131} labeled triiodothyronine to pregnant pigs.

An independent estimate of the lifespan of T-3* in the circulation of the fetus was approximated by administering T-3* to newborn guinea pigs, i.e., 24 hours or less old. Decay of 1 and 10 μg. doses was, as can be seen from Figure 9, only slightly faster than for T-3* which traversed the placenta, i.e., $T_{1/2}$ 7.6 and 9.2 hours respectively, compared with 10.0 hours.

TABLE 2. BODY CONTENT OF TOTAL I^{131} AND T-3* ONE HOUR AFTER INJECTION OF T-3* INTO PREGNANT PIG

	% of total activity recovered	% of total activity/gm. body wt.	% of dose as T-3*/gm. body wt.	Ratio of total body to plasma T-3* concentration
Mother	93	.093	.076	0.38
Fetus 1	1.0	.018	.0027	0.61
Fetus 2	0.9	.018	.0017	0.47
Fetus 3	1.0	.016	.0020	0.91
Fetus 4	0.9	.014	.0009	0.50

This small difference presumably reflects the relatively minor contribution of placental transfer to the fetal disposal curve. The obvious differences in experimental conditions and the lack of specific activity data do not justify calculation of the rate constant for placental transfer on the basis of comparison of fetal and newborn decay curves. On the other hand, using T-3 degradation by the newborn pig as a reasonable gross index of events in the

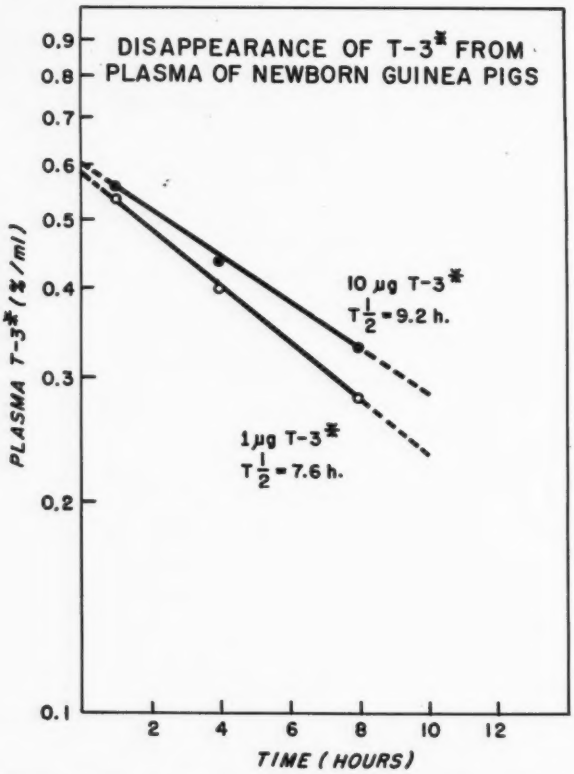

FIG. 9. Decay of labeled triiodothyronine in the plasma of newborn guinea pigs. Each point represents the mean value for four animals.

near term fetus, it would seem that fetal degradation is not the primary factor determining the observed fetal/maternal T-3* concentration gradient. The unlikelihood of this possibility is also indicated by the delay in equilibration with respect to total I^{131} (Fig. 7) and indirectly by failure of massive T-3 doses to modify the fetal/maternal ratios (Table 1).

Similarly, there is no evidence of a large fetal T-3 space to account for the low fetal T-3* concentrations. In the first place, it seems inconceivable that a 20 mg. dose of T-3 could distribute itself with a concentration

gradient of 0.05 by virtue of low specific activity on the fetal side. Secondly, the extrapolated volume of distribution of labeled T-3* (Fig. 8) is the same for the pregnant adult as for the newborns (Fig. 9). Finally and most directly, the total carcass analyses yielded concentrations lower than in plasma. This last observation also eliminates the possibility of pooling of T-3* in some extravascular fetal depot.

The very nature of the placenta as a massive surface for exchange rules out limited total penetration as a factor in the 0.05 fetal/maternal ratios. The results of the experiments with massive doses of carrier T-3 testify

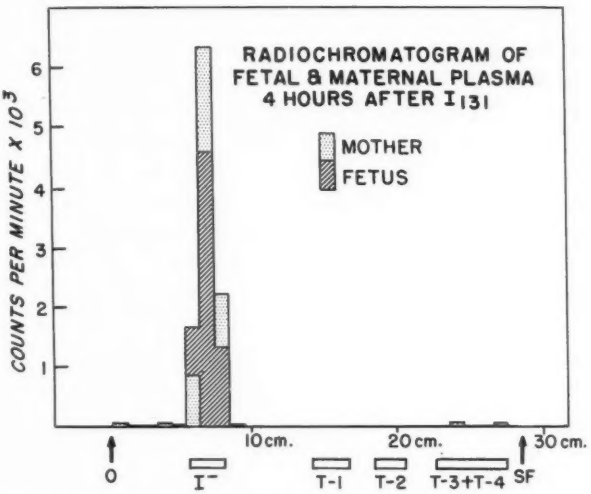

FIG. 10. Radiochromatogram of fetal and maternal plasma 4 hours after injection of I^{131} to perchlorate blocked pregnant pig.

directly to its efficiency in permitting maximal penetration of materials and eliminate this factor from further consideration.

It seems reasonable to conclude that the low concentration of T-3* in the fetus following its administration to the mother is due to relative non-diffusibility of the T-3 molecule or its protein carrier across the placenta. The small quantities of T-3* which *do* appear in fetal plasma are not due to exchange of I^{131} with I^{127} in preformed T-3 or to fetal incorporation of released I^{131} into its own hormone products despite pre-administration of $KClO_4$. These possibilities were ruled out by investigation of iodine compounds in the plasma of fetuses 4 hours after administration of I^{131} to the mother one-half hour after a 330 mg. dose of $KClO_4$. Figure 10 shows that only iodide is present in maternal and fetal plasma. In addition, there was no evidence of thyroxine in any maternal or fetal chromatograms after injection of T-3*.

COMMENTS

Production of massive thyroid enlargement in fetuses without effects on pregnant adult thyroids is in keeping with the concept of independence of the fetal pituitary-thyroid axis, as shown more directly by Nikitovich and Knobil (3) in the rat. It is reasonable to ascribe fetal goiters to entrance of perchlorate into the fetal circulation and not to maternal thyrotropin because there is no evidence of heightened thyrotropin activity in the mothers. The apparent extreme fetal sensitivity to iodine depletion by perchlorate reflects the intrinsic hyperactivity of the fetal thyroid as shown by its relatively large mass, comparative histology, and iodine concentrating ability. This apparent hyperfunction probably also applies to thyroid hormone production judging from the more rapid appearance (4) of radiothyroxine in fetal than in maternal thyroglobulin, and a conversion ratio which was higher in the fetal than in the maternal bovine circulation. It is not inconceivable that fetuses concentrate perchlorate and in doing so develop goiter more readily, but this possibility has not been investigated.

Administered T-3, on the other hand, is apparently effectively excluded from the fetus. This conclusion is based upon (a) the failure of large doses of T-3 to prevent or modify perchlorate induced fetal goiter and (b) the partition of labeled T-3* across the placenta with a fetal plasma concentration only one-twentieth that in the maternal circulation. Rapid degradation of T-3, though not responsible for the observed placental gradient, places further limitations on the concentration which can be achieved and maintained in the fetus for 24 hours following a single dose.

These observations are in keeping with the fate of labeled thyroxine administered to pregnant rabbits (5)[5] in that the fetal/maternal plasma ratio of labeled thyroxine, identified as the butanol soluble fraction, exceeded 0.054 in only one of the 23 rabbits. At four hours, practically all of the fetal radioactivity was present in the butanol soluble fraction, i.e., presumably as thyroxine. Barring species differences, this suggests that the major difference between administered thyroxine and T-3 from the point of view of access to the fetus is the more rapid degradation of the latter. This conforms to the known differences between T-3 and thyroxine turnover rates in rodents (6, 7) and implies that a dose of thyroxine, though equilibrated across the placenta in much the same fashion as T-3, survives longer and thereby exerts more pharmacologic activity. This difference probably accounts for the *partial* suppression of propylthiouracil induced fetal goiter in guinea pigs (8) when the mother received 25 μg. of dl-thyroxine daily. The same applies to the findings of Theresa (9) who noted microscopic involutional changes in fetal thyroids when pregnant rabbits

[5] The author is grateful to Drs. Hall and Myant for providing the opportunity to see their manuscript prior to its publication.

were given 0.05–0.1 gm. of thyroid powder. Reconsidering the data of Peterson and Young (8) and Theresa (9) in relation to the present study and the experiments of Hall and Myant (5), the weight of present evidence supports the original contention of Courrier and Aron (10) that administered thyroid hormones do not readily enter the fetal circulations of experimental animals. That this may also be the case in man is suggested by the report of goiter in the infant of a woman who received 500 μg. of thyroxine daily while taking propylthiouracil (11). The apparent similarities between the partition of thyroxine and triiodothyronine at the placental interface run somewhat counter to the concept of greater penetrability of trriodothyronine (6) and merits more direct comparative study.

Finally, the failure to observe a concentration gradient for iodide across the placenta in the present study suggests that perchlorate, like thiocyanate (1), interferes with its development.

SUMMARY AND CONCLUSIONS

Administration of $KClO_4$ to guinea pigs during the latter half of pregnancy resulted in large goiters in fetuses, but did not produce maternal goiter. In non-pregnant females thyroid enlargement occurred after 60 days of $KClO_4$ ingestion but not after 30 days, although some iodine depletion occurred over the shorter interval. Perchlorate-induced fetal goiter was not modified by simultaneous parenteral administration of *l*-triiodothyronine (T-3) in graded doses up to 32 μg. per day.

During the 24 hours after its administration, the concentration of I^{131} labeled T-3* in fetal plasma was only one-twentieth of the maternal concentration. Over this interval there was parallel decline in fetal and maternal T-3* concentration, corresponding to a biological half life of 10–11 hours. The ratio of the concentration of total I^{131} in plasma of fetuses to that of the mothers approached unity at 24 hours. Introduction of as much as 20 mg. of carrier did not modify the concentration gradient for T-3*, and there was no evidence of a large T-3 space to account for the observed discrepancy between maternal and fetal plasma concentrations of labeled T-3*. The biological half life of labeled T-3* in the plasma of newborn guinea pigs was of the same order as that of T-3* derived from the maternal circulation indicating that extremely rapid fetal degradation is not responsible for the fetal/maternal concentration gradient.

It is concluded that:

1) Perchlorate ion crosses the placenta and is a potent goitrogen in the guinea pig fetus by virtue of the sensitivity of the intrinsically hyperplastic fetal thyroid to iodine depletion.

2) Triiodothyronine administered to the pregnant guinea pig is ineffective in fetal goiter prevention because the placenta restricts its entry into the fetus, and it is rapidly degraded.

Acknowledgments

Mr. Harris Goldberg provided able technical assistance. The author is also indebted to Drs. John B. Stanbury, Gordon L. Brownell and members of the Thyroid Clinic of the Massachusetts General Hospital for suggestions and criticism.

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CHANGES IN BONES AND JOINTS OF UNDERFED MICE BEARING ANTERIOR HYPOPHYSEAL GRAFTS[1]

RUTH SILBERBERG AND MARTIN SILBERBERG

Snodgras Laboratory, City Hospital Division, and Department of Pathology, Washington University, School of Medicine, Saint Louis, Missouri

IN MICE and guinea pigs, transplants and extracts of anterior hypophysis promoted skeletal growth and development; they also increased the incidence and advanced the onset of degenerative joint disease, the active principle being the somatotrophin fraction (1–3). Since under the influence of STH food consumption is increased (4), and since on the other hand the development of osteoarthritis was retarded in underfed rats (2), it seemed of interest to determine, whether or not the skeletal effects of anterior hypophysis were related to increased food intake. For this purpose, anterior hypophyses were transplanted into underfed mice, and the changes occurring in the long bones and joints were studied.

MATERIAL AND METHODS

One hundred and thirty-five male mice of the closely inbred strain A one month of age and weighing 10 to 14 gm. with an average of 12 gm. at the beginning of the experiments were used. Seventy-nine of these mice received transplants of four anterior hypophyses from animals closely related (brothers, sisters or cousins) to the recipients, and three to four months old. The method employed has been fully described elsewhere; surviving grafts have been repeatedly illustrated, and their fate as well as the advantages and disadvantages of the method have been discussed previously (3).

The animals had water available at all times and were fed a stock diet of Purina laboratory chow in amounts restricted to still permit slow gain in body weight (1 gm./week or less) during the period of growth, until they had reached an arbitrarily chosen weight limit of 22 gm., which is about one third lower than the weight attained by controls fed *ad libitum*. This weight was reached after periods varying from 14 to 45 weeks with a mean of 29 weeks. Subsequently, food restriction was adjusted so that adult mice did not gain weight beyond 22 gm. Occasionally an animal would gain a few grams above the limit. This was corrected within a week by further decreasing the amount of food offered. In order to assure each individual mouse an adequate food supply and to prevent aggressive animals from taking food away from others, the mice were kept in individual compartments of partitioned enamel pans measuring $12\times7\times4\frac{1}{2}''$ four animals to a pan. The amount of laboratory chow permitting an average growth of no more than 1 gm./week was found to be about $2\frac{1}{2}$ gm./day. In adult mice, a weight of about 22 gm. was maintained by providing about 3 gm./day of chow for each animal through life as compared to $4\frac{1}{2}$–5 gm./day for *ad libitum* fed controls.

Received June 28, 1956.

[1] The investigation was supported by Grant A-22(C5) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

The mice remained on this regimen until they died or had to be killed because of illness. At necropsy, the legs were removed for microscopic examination in addition to tissues suspect of being grafted hypophysis.

OBSERVATIONS

Mortality and weights: Non-grafted mice tolerated the restriction of food comparatively well. Morbidity and mortality were low; only three of 56 mice died of inanition before the age of 9 months; four mice lived as long as 27 months which was about the same life span as that of controls fed *ad libitum*. The mean life span of all mice of the underfed group was 15 months as compared to 18.6 months of untreated mice.

The weight gain of underfed mice bearing hypophyseal grafts was more irregular than that of the underfed non-grafted animals. The limit of 22 gm. was reached after an average of 34 weeks. These mice had a high mortality early in life. Twenty-one died before the age of three months, and six additional ones before the age of eight months. These 27 animals are not included in this report. The mortality was not related to the procedure of transplantation; death was due to inanition as was indicated by the microscopic appearance of the bones which showed loss of primary spongiosa and atrophy of the bone marrow.

MICROSCOPIC FINDINGS

(*a*) *Epiphyseal growth zones at the upper end of the tibia*

Controls fed ad libitum: At the age of 12 months, the growth zones consisted of inactive cartilage cells with intense calcification and many plugs of ossification and degeneration. A bony lamella delimited the remnants of the cartilage from the metaphysis, and only a few short bony spicules could occasionally be detected. At 18 months of age, the growth zones were hyalinized or ossified (Fig. 1).

Underfed mice: The changes were essentially similar to those described previously for underfed guinea pigs and rats (2). Epiphyseal growth and development were retarded; degeneration of cartilage and epiphyseo-

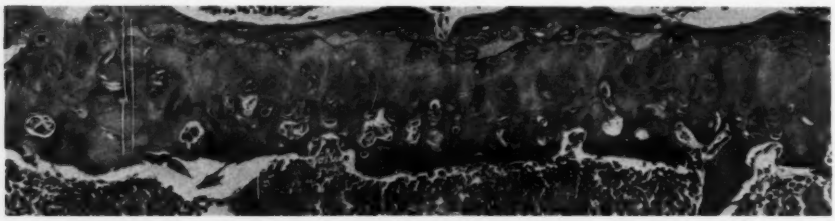

FIG. 1. Epiphyseal growth zone at the upper end of the tibia of an 18-month-old mouse fed *ad libitum*. ×140. The epiphyseal cartilage is inactive and hyalinized; near the left margin of the picture an acellular plug of degenerated cartilage is seen. The cartilage is sealed off the metaphysis by a bony lamella.

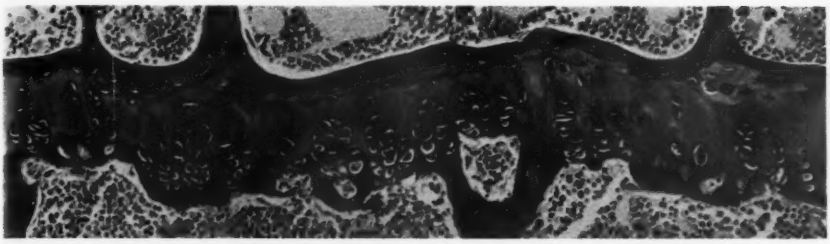

FIG. 2. Epiphyseal growth zone at the upper end of the tibia of an 18-month-old underfed mouse. ×140. The structural age of the growth zone is younger than that of the control fed *ad libitum* demonstrated in Figure 1. The epiphyseal plate contains short columns of cartilage cells undergoing ossification; a good deal of hyaline matrix is seen between the cartilage cell columns. The bony lamella delimiting the cartilage from the metaphysis is thin.

diaphyseal union were markedly delayed. In 12-month-old mice, the growth zones still contained growing cartilage undergoing endochondral ossification. The epiphyseal plates were 70–90 μ wide, and in the single cartilage cell row as many as 5 to 7 columnar and one or two hypertrophic cartilage cells were seen; metaphyseal trabeculae were still present, and the cartilage had not yet been sealed off the diaphyseal cavity. Thus, the development of the growth zones was delayed by about one half year as compared to that of *ad libitum* fed controls. At 18 months of age, the growth zones of underfed mice still showed short cartilage cell rows with markedly delayed regression (Fig. 2).

Underfed mice bearing hypophyseal transplants: Epiphyseal growth, development and ageing were accelerated to a degree that would sometimes exceed that seen in untreated controls fed *ad libitum*. At the age of 18 months, the epiphyseal cartilage was in an advanced state of inactivation with wide perforations of the epiphyseal plate (Fig 3).

Figures 1–3 illustrate findings in mice of the various groups at the age of 18 months.

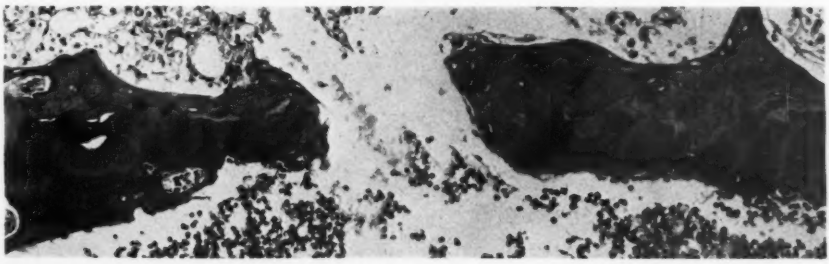

FIG. 3. Epiphyseal growth zone at the upper end of the tibia of an 18-month-old underfed mouse bearing hypophyseal grafts. ×140. The epiphyseal plate is in an advanced state of inactivation; only a few atrophic cartilage cells are recognizable. The perforation of the growth zone indicates epiphyseo-diaphyseal union.

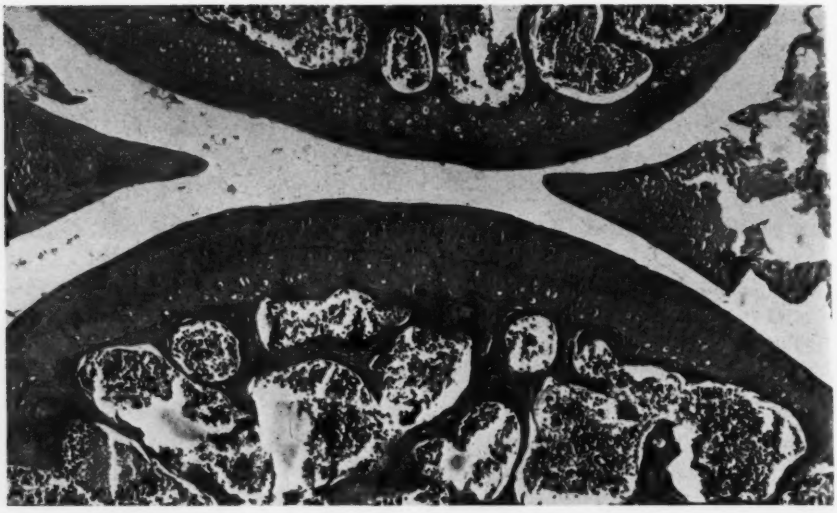

FIG. 4. Unchanged articular surface of the knee joint. ×85. Tibia at the lower, femur at the upper end of the illustration, menisci at each side. The articular surfaces are smooth and covered by well organized regular cartilage.

(*b*) *Joints*

As in former studies (3), the articular changes were graded into age changes and osteoarthritic lesions. Age changes consisted of hyperplasia and hypertrophy of the articular cartilage with or without minor regressive changes but without fraying of the surface or changes in other articular structures. In arthritic joints, hypertrophic or ulcerative lesions occurred alone or in combination with each other (Figs. 4–7). Cartilage, synovialis and ligaments may be involved: The cartilage is hyperplastic and hypertrophic and shows various types of degeneration and ulceration; the syno-

FIG. 5. Ulcerative type of osteoarthritis showing a shallow defect of the cartilage covering the tibia. ×85. At the base of the ulcer, the bone is eburnized.

vialis proliferates and undergoes mucoid swelling, and in the ligaments mucoid change, cartilagination or ossification are noted; in the bone marrow foci of fibrosis and cyst formation are seen.

In Tables 1–4, the incidences of age changes and of osteoarthritis in mice of the various experimental and control groups are given. The animals were divided into three age groups from 8 to 12, 13 to 18, and 19 and more months respectively so as to permit a better evaluation of the development of the joint lesions with advancing age.

FIG. 6. Hypertrophic type of osteoarthritis at the femur. ×85. The articular cartilage varies in thickness and shows irregular outgrowths. The cartilage cells undergo hyperplasia and hypertrophy with some areas showing basophilia and others loss of cells.

In underfed mice, the incidence of osteoarthritis was lower, and the lesions appeared later than in the *ad libitum* fed controls. Not only was osteoarthritis less frequent but it was by far less severe than in the controls fed *ad libitum*. Ulcerations in particular were unusual in mice of the underfed group and, if present at all, they consisted of superficial erosions rather than deep ulcers with eburnated floors and marginal outgrowths. Proliferation of cartilage as well as changes in the ligaments were likewise much less pronounced in the underfed than in the *ad libitum* fed animals.

On the other hand, in underfed mice carrying hypophyseal grafts, the over-all incidence of osteoarthritis (53.8%) was higher than that found in underfed non-grafted mice (25%), and approximately that seen in mice fed *ad libitum* (48.9%). In the first age group, 26.6% of the grafted and only 9.5% of the non-grafted underfed mice had degenerative joint disease, in the second age group the corresponding data were 24% for the non-grafted and 60.6% for the grafted mice; in the third age group, however, there was only a minor increase of the joint disease in grafted mice, while

FIG. 7. Advanced hypertrophic type of osteoarthritis involving the ligaments. ×85. The ligaments show cartilagination, fibrillation and liquefaction of the ground substance. The adjacent articular cartilage and that of the menisci (top of the illustration) undergoes hyperplasia, hypertrophy and focal liquefaction.

60% of the non-grafted mice now showed arthritis. In grafted mice the increased incidence was associated with increased severity and accelerated onset of the joint disease. The effect of hypophyseal grafts in underfed mice was less marked than in mice fed *ad libitum*. Of the latter, 80% had osteoarthritis, and most of the lesions were severe.

TABLE 1. ARTICULAR FINDINGS IN NORMAL CONTROLS

Age group	Mice No.	Mean age, months	Articular findings (per cent)		
			No changes	Age changes	Osteoarthritis
8–12	6	10.0	66.7	16.2	16.1
13–18	15	15.5	20.0	46.7	33.3
19–23	22	20.9	9.1	22.7	68.2
8–23	43	17.4	20.9	30.2	48.9

TABLE 2. ARTICULAR FINDINGS IN UNDERFED MICE

Age group	Mice No.	Mean age, months	Articular findings (per cent)		
			No changes	Age changes	Osteoarthritis
9–12	21	10.5	33.3	57.1	9.6
13–18	25	16.5	16.0	60.0	24.0
19–27	10	23.7	10.0	30.0	60.0
9–27	56	15.0	21.4	53.6	25.0

TABLE 3. ARTICULAR FINDINGS IN UNDERFED MICE BEARING HYPOPHYSEAL GRAFTS

Age group	Mice No.	Mean age, months	Articular findings (per cent)		
			No changes	Age changes	Osteoarthritis
8–12	15	9.8	6.7	66.7	26.6
13–18	24	14.6	8.3	29.1	62.6
19–24	13	20.6	7.7	23.1	68.2
8–24	52	15.0	7.7	38.5	53.8

TABLE 4. ARTICULAR FINDINGS IN *ad libitum* FED MICE BEARING HYPOPHYSEAL GRAFTS

Age group	Mice No.	Mean age, months	Articular findings (per cent)		
			No changes	Age changes	Osteoarthritis
13–29	10	17.5	0.0	20.0	80.0

DISCUSSION

In male mice of strain A, underfeeding by approximately one third of the *ad libitum* food intake retarded epiphyseal development and decreased the incidence and severity of degenerative joint disease as compared to that found in untreated controls. Regressive changes that precede ossification, ossification itself, and resorption of the epiphyseal cartilage were lagging behind the corresponding processes in controls fed *ad libitum*. On the other hand, in underfed mice anterior hypophyseal grafts promoted development and ageing of the epiphyseal growth zones sometimes even beyond the degree reached in *ad libitum* fed controls.

In the joints, conditions were similar to those observed in the epiphyseal

growth zones. The inhibited growth and the delayed degeneration and resorption of the articular cartilage resulted in an attenuation of osteoarthritic changes. These observations are in keeping with results obtained previously in rats (2), and demonstrate again the similarity in the physiologic response of epiphyseal and articular cartilages to certain promoting or retarding influences.

The mechanisms by which underfeeding acts on the skeleton are unknown. One may have to deal with a direct effect on the cells; there may also be indirect effects exerted by the hypophysis. The latter is known to become hypofunctional in undernourished individuals thus causing decreased growth of cartilage and bone (2). Moreover, there is the possibility that the adrenals stimulated by underfeeding (5, 6) may increase the output of antianabolic glucocorticoids and thus inhibit growth of cartilage (7, 8).

The basic mode of action of the hypophyseal grafts in underfed animals is apparently the same as in animals fed *ad libitum*, and similar to that exerted by hypophyseal extracts or purified hypophyseal growth hormone (9–12). Presumably, by producing STH, the transplanted hypophyses promoted epiphyseal and articular growth and degeneration, and therefore, compensate or overcompensate for the retardation of these changes caused by underfeeding. On the other hand, hypophyseal grafts assert themselves to a lesser degree in the underfed animal than in *ad libitum* fed controls. This may be due to an impairment of hormone production by the grafts or to an inadequate supply of building stones in the underfed animal.

SUMMARY

In male mice of strain A, restriction of food intake to about two thirds of the amount consumed *ad libitum* retarded epiphyseal development, and decreased the incidence and severity of degenerative joint disease. Transplants of anterior hypophysis obtained from closely related donor animals reversed these changes accelerating skeletal development and increasing the incidence of degenerative joint disease. The effect of the anterior hypophyseal grafts—also observed in controls fed *ad libitum*—was thus partly independent of the amount of food consumed. However, in underfed animals the transplants were less effective than in controls fed *ad libitum*.

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THE EFFECTS OF CARBUTAMIDE (BZ-55) ON BLOOD SUGAR LEVELS OF DEPANCREATIZED DOGS GIVEN INSULIN[1]

I. B. FRITZ,[2] M. WEINSTEIN, J. V. MORTON AND R. LEVINE

Department of Metabolic and Endocrine Research, Michael Reese Hospital, Chicago, Illinois

SEVERAL recent reports (1, 2, 3) have indicated that administration of BZ-55 (p-amino-benzene-sulphonamide-n-butyl-urea) results in a fall of blood sugar of normal human subjects and various experimental animals. This material does not lower the blood sugar of pancreatectomized dogs, rabbits or alloxanized rats, and results in hypoglycemia only in certain human diabetics. On the basis of evidence obtained both *in vitro* and *in vivo*, Mirsky *et al.* (4, 5, 6) have put forward the hypothesis that the sulfonyl hypoglycemic derivatives act by inhibiting hepatic insulinase. Further, these workers (4) have postulated that human diabetes is not necessarily a resultant of insulin deficiency but may be rather a consequence of increased insulin destruction, presumably by the hepatic insulinase system.

While the available evidence strongly suggests that the sulfonyl derivatives act to increase the net amount of functioning insulin, the mechanism by which this is achieved remains unknown. In the present report, experiments have been designed to test the possibility that BZ-55 acts by inhibiting insulinase *in vivo*. Data from acute experiments indicate that BZ-55 does not augment the action of insulin administered to pancreatectomized dogs. It therefore appears that BZ-55 does not act primarily by inhibiting destruction of exogenous insulin.

METHODS

Mongrel dogs of both sexes were pancreatectomized and maintained for approximately a week post-operatively. During this time, the dogs received injections of insulin (5 units, regular) twice a day. The last dose of insulin was given 16 hours prior to the beginning of the experiment. They were fed eggs, milk, and raw pancreas in addition to usual dog rations. Animals were then fasted overnight and were anesthetized with nembutal. Insulin was injected intravenously, either via an arm vein or via a tributary of the portal, in dosages varying between 0.02 units per kg. per hour to 0.20 units per kg. per hour in different dogs. The route of administration did not seem to be of importance in these experiments.

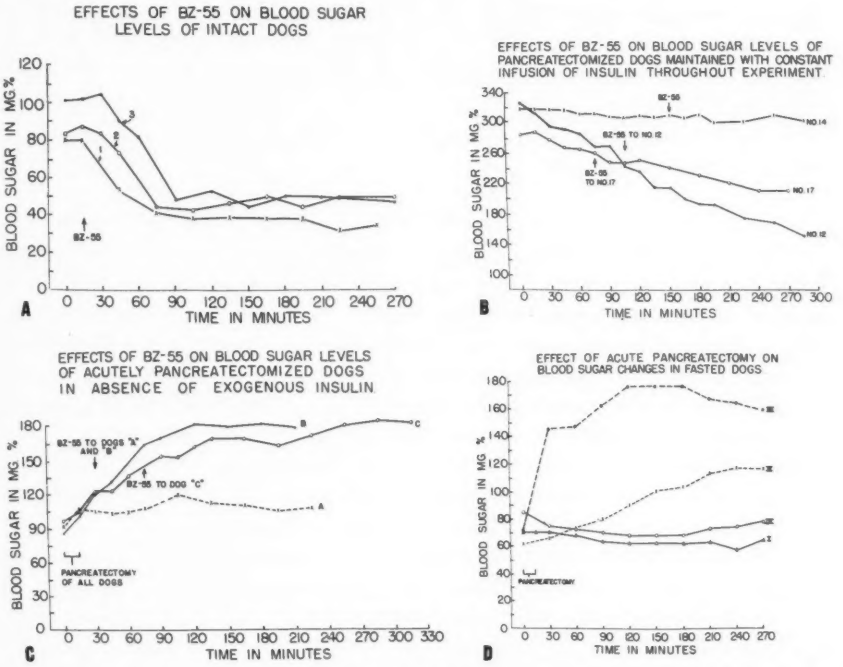
Received July 5, 1956.

[1] This work was partially supported by grants from the Eli Lilly Company and the Upjohn Company.

[2] Present address: University of Michigan, Department of Physiology, Ann Arbor, Michigan.

Blood samples were drawn from the femoral artery at 15 minute intervals for over an hour to establish a trend of blood sugar concentration changes. Blood glucose was determined colorimetrically by the method of Nelson (7). At this time, one gram of BZ-55[3] was administered in a single intravenous injection, and the blood sugar concentrations were followed.

In control experiments, normal fasted dogs were similarly treated with BZ-55. In other experiments, dogs were pancreatectomized immediately prior to being subjected to the administration of BZ-55. In all cases, blood sugar changes were followed for 3 to 4 hours following BZ-55 injection.


EFFECTS OF BZ-55 ON BLOOD SUGAR LEVELS OF INTACT DOGS
BLOOD SUGAR IN MG.%
TIME IN MINUTES
BZ-55
A
EFFECTS OF BZ-55 ON BLOOD SUGAR LEVELS OF PANCREATECTOMIZED DOGS MAINTAINED WITH CONSTANT INFUSION OF INSULIN THROUGHOUT EXPERIMENT
BZ-55
BZ-55 TO NO.12
BZ-55 TO NO.17
NO.14
NO.17
NO.12
B
EFFECTS OF BZ-55 ON BLOOD SUGAR LEVELS OF ACUTELY PANCREATECTOMIZED DOGS IN ABSENCE OF EXOGENOUS INSULIN
BZ-55 TO DOGS "A" AND "B"
BZ-55 TO DOG "C"
PANCREATECTOMY OF ALL DOGS
C
EFFECT OF ACUTE PANCREATECTOMY ON BLOOD SUGAR CHANGES IN FASTED DOGS
PANCREATECTOMY
D

FIGS. 1A–D. The numbers or letters which label each curve refer to results obtained with an individual animal, with 13 representative dogs being selected from the total of 23 successful experiments performed. At the time indicated by arrows one gram of BZ-55 was administered intravenously. In Figures 1C and 1D, each dog was pancreatectomized during the time interval indicated by the bracket. The weights of the dogs ranged from 7 to 15 kg.

RESULTS

Representative experiments graphed in Figure 1A indicate that administration of BZ-55 to normal dogs resulted in a fall in blood sugar which was sustained for several hours. This agrees well with the findings of previcus investigators (1, 2, 3).

[3] The BZ-55 was dissolved in 1.0 N NaOH until the solution was saturated, and this was then diluted to a final concentration of 10% BZ-55. We wish to express our thanks to Eli Lilly and Company for a generous supply of BZ-55.

In contrast, representative experiments graphed in Figure 1B indicate that administration of BZ-55 to pancreatectomized dogs maintained with constant intravenous infusions of insulin did not result in an enhanced fall in blood sugar. This was the case both in those animals given just sufficient insulin to maintain the blood sugar at a constant level, and also in those animals given enough insulin to produce a steady decline in blood sugar levels.

In Figure 1C it can be seen that dogs pancreatectomized immediately before administration of BZ-55 also failed to develop hypoglycemia during the time periods studied after the injection of this agent. The controls for these latter experiments are graphed in Figure 1D, where it can be seen that the blood sugar levels either rise or stay constant immediately after pancreatectomy. These changes were variable regardless of whether BZ-55 was administered or not.

DISCUSSION

The results clearly indicate that a single dose of BZ-55 injected into normal dogs had rapid hypoglycemic effects, but these effects could not be elicited in pancreatectomized dogs treated with constant infusions of insulin. These data strongly suggest that BZ-55 does not act acutely in normal animals by inhibiting insulin destroying agents. It is possible that insulin administered exogenously is not treated in the same fashion as endogenous insulin, and that BZ-55 prevents the destruction only of "natural" insulin. However, this does not seem to be the case, since acutely pancreatectomized animals, which presumably still have some endogenous insulin left, did not respond to BZ-55 administration either.

In accordance with the results of these experiments, BZ-55 had no effects on glucose disappearance in eviscerated dogs maintained on constant infusions of glucose with or without insulin. These results are not tabulated, because the acute pancreatectomy experiments are more readily interpretable.

The site of action of BZ-55 is unknown. Several recent reports have demonstrated an effect *in vitro* on inhibition of hepatic insulinase. However, low concentrations of BZ-55 failed to produce this inhibition (8). It is unlikely that BZ-55 acts by inhibition of production or secretion of glucagon, since a hypoglycemic action cannot be evoked in the alloxan diabetic animal by the administration of sulfonyl derivatives (2). The possibility remains open tht the β-cells of the Islets of Langerhans directly respond to certain sulfonyl derivations with an increased production and/or secretion of insulin. This had been suggested in 1944 by Loubatieres (9, 10, 11) who published experiments which indirectly indicated that certain other sulfonyl-derivative compounds elicited the secretion of insulin. These findings need confirmation and extension, possibly in hepatectomized preparations with pancreas and pancreatic blood supply intact.

SUMMARY

Acutely pancreatectomized dogs and pancreatectomized dogs maintained with constant infusions of insulin failed to respond to single intravenous injections of BZ-55. These findings indicate that BZ-55 does not produce its hypoglycemic effects by inhibiting destruction of insulin *in vivo*. The significance of the findings was discussed.

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THE INFLUENCE OF TESTOSTERONE ON THE GROWTH OF THE URINARY BLADDER[1]

CHARLES D. KOCHAKIAN, GERALD ENDAHL, AND JANE AUSTIN

Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma

THE observation that androgens influenced the growth of the kidney (1, 2, *cf* 3) suggested that the growth of other portions of the urinary tract also might be influenced by androgens (4). Therefore, studies were conducted on the influence of androgens on the urinary bladder. Since the mouse is the most sensitive animal to the renotrophic effect of the androgens, this animal was chosen for this study.

METHODS

The mice were of the Swiss strain (National Laboratories, Creve Coeur, Mo.). They were kept on wood shavings in groups of 2 to 4 in glass jars, and were fed Rockland Mouse Diet *ad libitum*. Body weights were determined three times per week.

Castration was performed under ether anesthesia. The testosterone[2] was administered as a cylindrical pellet of about 15 mg. implanted subcutaneously (5).

The animals were killed by severing the spinal cord at the base of the skull and bleeding from the neck. The organs were weighed on an appropriate Roller-Smith torsion balance. The urinary bladders were cut and blotted on paper towels prior to weighing. They were then dried in an electric oven at 90–95° for 48 hours to determine moisture content. The dried samples were analysed for nitrogen by the micro-Kjeldahl procedure.

RESULTS

Effect of Age and Castration: The urinary bladder progressively increased in weight until about 80 days of age after which it apparently remained constant (Fig. 1).

Castration decreased the rate of increase of the bladder weight with age (Fig. 1).

The nitrogen content of the bladders and also the moisture (not shown) changed in parallel with the weight (Fig. 1).

Effect of Testosterone: Testosterone produced an increase in bladder weight with a concomitant change in water and nitrogen (protein). (Table 1) The changes were progressive (Fig. 2) and after 41 and 45 days of treatment, the bladder had almost doubled in size.

Received July 12, 1956.

[1] This investigation was aided by a grant from the American Heart Association.

[2] The testosterone was generously provided by Syntex, S. A.

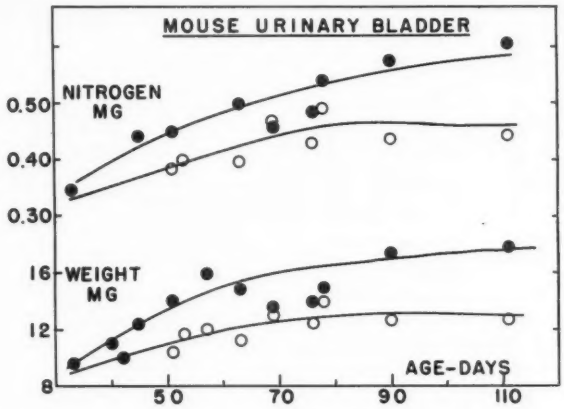

FIG. 1. Effect of age and castration on the weight and nitrogen content of the urinary bladder of the mouse. Normal ●—●, castrated ○—○. Each value represents the average of 6 mice. Castration was performed at 30 to 39 days of age.

The initiation of treatment with testosterone at the time of castration or 28 days later yielded practically identical results.

The seminal vesicles and prostates and the kidneys showed the expected (3) increases in weight after testosterone treatment.

TABLE 1. EFFECT OF TESTOSTERONE (T)[1] ON THE WEIGHT AND NITROGEN AND WATER COMPOSITION OF THE URINARY BLADDER OF THE MOUSE[2]

	Body wt. (gm.)	T abs'd (mg.)	T abs'd (days)	Kidney (mg.)	Sem. ves. +pros. (mg.)	Urinary bladder Wt. (mg.)	Urinary bladder H_2O %	Urinary bladder N %
T. pellet implantation at time of castration[3]								
Castrate	23.0	—	—	267	16	12 ± 1.2	76.6	3.57
Castrate + T	22.0	4.8	14	366	253	14 ± 0.8	76.1	3.79
Castrate	24.2	—	—	254	17	12 ± 0.9	77.2	3.42
Castrate + T	26.0	6.9	21	423	292	17 ± 1.3	77.4	3.35
Castrate	26.5	—	—	264	18	14 ± 1.1	79.2	3.50
Castrate + T	26.7	12.4	41	472	397	26 ± 2.3	79.2	3.04
T. pellet implanted 28 days after castration[4]								
Castrate	26.0	—	—	271	18	13 ± 1.1	78.2	3.31
Castrate + T	31.4	2.3	10	483	204	17 ± 3.0	78.6	3.47
Castrate	26.4	—	—	267	19	13 ± 0.7	76.7	3.36
Castrate + T	31.5	5.8	24	540	461	18 ± 1.6	75.4	3.18
Castrate	31.2	—	—	290	22	13 ± 0.7	76.0	3.40
Castrate + T	34.1	10.2	45	616	604	24 ± 2.2	74.2	3.56

[1] A pellet (approx. 15 mg.) of testosterone was implanted subcutaneously.
[2] The values are the averages of 6 mice per group. The standard error of the bladder weights are given.
[3] Castration was performed at 30 days of age.
[4] Castration was performed at 39 days of age.

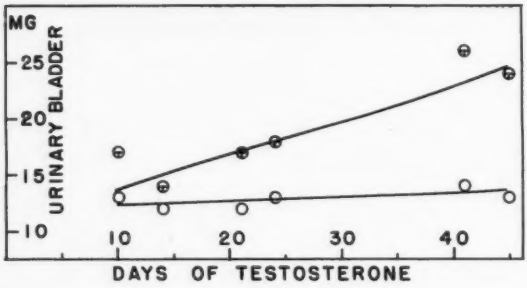

FIG. 2. Effect of duration of treatment with testosterone on the weight of the urinary bladder of the castrated mouse. See Table 1 for details. Control, ○—○; testosterone treated, ⊖—⊖.

DISCUSSION

These results indicate that testosterone plays a role in the growth and function of the urinary bladder. This is in keeping with the growth stimulating effects of the androgens on the kidney and the muscles of the body (*cf* 6) and provides an explanation for the general clinical observation that the administration of testosterone improves the tone of the urinary tract in elderly men.

The urinary bladder now becomes recognized as another tissue of the many which are influenced by the androgens (*cf* 3).

SUMMARY

Castration produces a decrease in the growth of the urinary bladder of the mouse. The administration of testosterone to the castrated mouse results in a progressive increase in bladder weight. These changes are accompanied by proportionate changes in nitrogen (protein) and water content of the bladder.

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METABOLISM OF L-THYROXINE AND L-3:5:3′-TRIIODOTHYRONINE BY BRAIN TISSUE PREPARATIONS[1]

JAMSHED R. TATA[2], J. E. RALL[3] AND RULON W. RAWSON

Department of Clinical Investigation of the Sloan-Kettering Institute for Cancer Research, New York, New York

THYROID gland preparations have been known for many years to have a marked effect on the development and function of the central nervous system (1). More recently, the thyroid hormones, *l*-thyroxine and l-3:5:3′-triiodothyronine, have been shown to increase the excitability of the brain, as measured by the threshold for electric shock seizures (2, 3). Both substances also influence the electrolyte distribution and in particular, the distribution of sodium in the brain tissue (3). Absence of the thyroid gland has marked effects on the electroencephalogram and causes profound changes in cerebral vascular resistance and blood flow (4). Moreover, changes in mental activity, mood and even psychosis may be associated with either over- or under-activity of the thyroid (5, 6, 7, 8). There is, however, no change in oxygen consumption in brain tissue removed from either thyroidectomized or hyperthyroid rats (9) although hyperthyroidism may effect a slight change in succinoxidase activity (10).

Small amounts of thyroxine and triiodothyronine can be detected in the central nervous system itself, using hormones labeled with radioiodine (11, 12, 13). Even this amount may be significant in view of the blood-brain barrier which exists for most substances of physiological activity (14). Recently, thyroxine and an unidentified iodinated substance, considered as a metabolite of the hormone, have been demonstrated in human cerebrospinal fluid (15). The metabolite was not found in blood and hence it seems possible that it was formed in the central nervous system itself. Therefore, the present study was undertaken to examine the activity of

Received July 19, 1956.

[1] This investigation was supported in part by a research grant (C-2052) from the National Institutes of Health, Public Health Service. It was also supported in part by a grant-in-aid (EDC-20A) from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council. This work was undertaken during the tenure of a Damon Runyon Memorial Fund Grant. This work was also made possible by support from the United States Atomic Energy Commission under their Contract No. AT (30-1)-910.

[2] Visiting Research Fellow.

[3] Present address: National Institutes of Health, Bethesda, Maryland.

brain tissue in the metabolism of *l*-thyroxine and *l*-3:5:3′-triiodothyronine.[4]

METHODS AND MATERIALS

Tissue Preparations

Unanaesthesized adult male or female dogs (5–10 kg.) male or female rats (75–125 gm.) and two-week old male chicks[5] were sacrificed by exsanguination or decapitation. Whole brains were collected in crushed ice or frozen in dry ice and, in the case of the rat and chick, brains from 15–30 animals were pooled. Brain slices were cut free-hand from 0.5 to 1.5 mm. in thickness, and suspended in the bicarbonate-Ringer solution of Krebs and Henseleit (16) in the proportion of 1 gm. wet tissue weight/5 ml. suspension medium. Tissue suspensions were prepared by homogenization in a Waring blender run at a reduced voltage (30–40 v.), the concentration of the suspension being variable: 1 gm. tissue/3, 5, 10, 15 or 30 ml. medium. The modified composition of the brain suspension medium (pH 7.4) was (17):

0.12 *M* NaCl	0.0012 *M* $MgSO_4$
0.004 *M* KCl	0.025 *M* $NaHCO_3$
0.0004 *M* KH_2PO_4	0.0005 *M* $CaCl_2$

Incubation

Incubations were carried out at 37.4° C with continuous shaking of containers. Most incubations, unless specifically mentioned as otherwise, were carried out in an atmosphere of 5% CO_2–95% O_2. A 5% CO_2–95% N_2 mixture was used for anaerobic incubations. In the case of tissue suspensions or homogenates, aliquots were drawn from each flask at intervals varying from 15 minutes to 12 hours of incubation, while for slices one incubation flask was allotted to each time interval.

Substrates

L-Thyroxine and l-3:5:3′-triiodothyronine labeled with I^{131} in the 3′:5′ and 3′-positions respectively (average specific activity: 40–60 μc./μg.) were obtained from Abbott Laboratories (18). Prior to use, the purity of each sample was ascertained by paper chromatographic analysis employing at least two different solvent systems. Only samples containing less than 10% contaminating radioactivity were used. Care was taken to avoid the inclusion of any n-butanol in the substrate solutions as this solvent was found to interfere with tissue activity. Non-radioactive samples of l-thyroxine and l-3:5:3′-triiodothyronine were dissolved in the minimum quantity of 50% propylene glycol and then diluted with the isotonic suspension medium to obtain the appropriate specific activity.

Control experiments

Each new series of incubations was accompanied by a set of at least two of the following control incubations where the substrate was incubated with: 1) suspension medium alone, 2) tissue system with n-butanol (final concentration five per cent), and 3) boiled tissue system (20–30 minutes boiling). The very small change in the final composition

[4] The following abbreviations are used in the text: T_4 = thyroxine; T_3 = triiodothyronine; TETRAPROP = 3:5:3′:5′-tetraiodothyropropionic acid; TETRAC = 3:5:3′:5′-tetraiodothyroacetic acid; TETRAFORM = 3:5:3′:5′-tetraiodothyroformic acid; TRIPROP = 3:5:3′-triiodothyropropionic acid; TRIAC = 3:5:3′-triiodothyroacetic acid; TRIFORM = 3:5:3′-triiodothyroformic acid.

[5] Thanks are due to Babcock Farms, for the generous supply of chicks used in this work.

of radioactive components after incubation did not vary appreciably between the different controls and the final values were averaged.

Analytical

The brain slices were analyzed either separately from the incubation medium or after homogenizing them in the same medium. Aliquots for chromatography were applied directly to paper strips without prior extraction. Paper chromatography (Whatman No. 1) was used for both qualitative and quantitative determinations of radioiodinated compounds in each sample (19, 20).

At least two of the following solvent systems were used for the analysis of any one sample:

1) n-butanol-acetic acid-water (78:10:12, v/v)
2) n-butanol-dioxane (80:20, v/v) saturated with 2N NH_4OH
3) collidine-water (100:35.5, v/v) in atmosphere of NH_3
4) ter-amyl alcohol saturated with 2N NH_4OH.

Each sample for chromatographic analysis was supplemented with 10–25 μg. of monoiodotyrosine, diiodotyrosine, 3:5:3′-triiodothyronine and thyroxine as carriers. The final positions of these was revealed by ninhydrin or the Pauly reaction. In some experiments, 25 μg. of each of the propionic, acetic and formic acid analogues of thyroxine and 3:5:3′-triiodothyronine were also added as carriers.[6] Added iodide (5 μg.) was revealed with 0.5% $PdCl_2$. The radioactivity in each chromatographic strip after development was determined with an automatic recording counting rate meter. The percentage distribution of radioactivity in the different iodinated components on the paper strips was determined by measuring the area occupied by each I^{131} peak, previously shown to be proportional to the activity on the strip.

Paper electrophoresis (Whatman No. 3) in barbital buffer at pH 8.6 or in 0.2 *M* $(NH_4)_2CO_3$ buffer at pH 9.0 was also employed, to distinguish between iodothyronines and their alanine side-chain derivatives. The techniques have been described elsewhere (20, 21).

As inorganic iodide was found to be a consistent and, in most cases, the major iodinated metabolite after incubation, periodic batches of samples were analyzed for their radioiodide content by a method previously described (22). This was done by the oxidation of radioiodide (after the addition of 50–100 μg. of stable iodide and organic iodinated compounds as carriers) by Fe^{+++} ions. The elemental iodine was extracted by CS_2 and absorbed in a $Na_2S_2O_3$ solution before measurement of I^{131}. Good agreement was found in the results obtained with the two different methods used for the estimation of the fraction of I^{131} in the form of iodide.

RESULTS

Brain Slices and "Concentrated" Suspensions

In the preliminary experiments, the tissue concentration for both slices and homogenates was in the range of one gm. wet wt./3 ml. of suspension medium. Substrate concentrations were varied from 10^{-5} *M* to 5×10^{-9} *M*. Table I summarizes the analytical data on dog brain incuba-

[6] We are very grateful to the members of the staff of Warner-Chilcott Laboratories, Morris Plains, N.J., for having supplied us with all the derivatives of the alanine side-chain of thyroxine and l-3:5:3′-triiodothyronine used in this work.

tion samples examined after three hours of incubation at concentrations of thyroxine and triiodothyronine much below the level of iodine found in the serum of the same animals. Normal dog serum iodine is about 2.5 μg.% or, if it is all thyroxine, about 5×10^{-8} *M*. Rat and human serum contains about 5 μg.% organic iodine, or, if it is all thyroxine, a concentration of about 10^{-7} *M*.

Definite degradation of both the hormones is noticed in all the samples except in controls where no change was produced. The major portion if the radioactivity was transferred from the substrate to the fraction identified as inorganic iodide and less than 5% of I^{131} could be detected in

TABLE 1. DEGRADATION OF L-THYROXINE AND L-3:5:3′-TRIIODOTHYRONINE BY "CONCENTRATED" TISSUE PREPARATIONS (1 GM. TISSUE/3ML. MEDIUM) OF DOG BRAIN. (INCUBATION TIME 3 HRS.)

Tissue preparation	Incubation	*l*-thyroxine, 8×10^{-9} *M*				*l*-triiodothyronine 8×10^{-9} M			
		% total I^{131} distributed as*				% total I^{131} distributed as*			
		T_4	I^-	O†	Unidentified	T_3	I^-	O†	Unidentified
Controls	Aerobic	85.6	12.1	—	2.3	87.6	9.2	+	1.0
	Anaerobic	83.5	13.4	1.0	2.1	91.1	8.7	+	—
Slices	Aerobic	26.9	69.3	—	2.2	28.3	65.8	1.5	1.6
	Anaerobic	25.7	68.6	+	4.4	32.5	63.6	2.1	1.8
Suspension	Aerobic	15.3	83.8	—	+	21.4	78.3	—	+
	Anaerobic	19.7	77.9	+	2.0	25.6	72.3	+	1.5

* + = less than 1%
† O = Fraction of I^{131} immobile in chromatography.

any other component outside of the unchanged thyroxine or 3:5:3′-triiodothyronine. Almost the same pattern of I^{131} distribution is found irrespective of whether the tissue was homogenized or sliced and under aerobic or anaerobic conditions. The presence of either glucose or PO_4 did not alter the results. Similar results were obtained with both rat and chick brain preparations. In all cases the rate and extent of degradation were considerably diminished near concentrations of 5×10^{-6} to 10^{-5} *M* thyroxine or 3:5:3′-triiodothyronine.

"Dilute" Suspensions

It was hoped that a dilution of the tissue system would lead to a retardation in the formation of iodide and detection of possible short-lived intermediate substances. The most usual tissue concentration used was 1 gm. wet wt./15 ml. of suspension medium and this concentration will hereafter be referred to as "dilute." Analytical data obtained at one, three and six hours after incubation using "dilute" dog and rat brain suspensions are compared in Table 2.

TABLE 2. DEGRADATION OF L-THYROXINE AND L-3:5:3′-TRIIODOTHYRONINE IN "DILUTE" SUSPENSION (1 GM. TISSUE/15 ML. MEDIUM) OF DOG AND RAT BRAIN

Animal	Hours of incubation	*l*-thyroxine, 1.6×10^{-9} *M*				*l*-triiodothyronine, 1.8×10^{-9} *M*			
		% total I^{131} distributed as				% total I^{131} distributed as			
		T_4	I^-	O	Unidentified	T_3	I^-	O	Unidentified
Dog	1	58.0	23.3	–	18.7	69.2	22.0	1.5	6.9
	3	45.6	44.0	+	9.0	55.2	39.1	–	5.7
	6	41.0	54.7	1.0	3.3	54.4	42.6	2.0	1.2
Controls (Dog)	6	89.3	9.5	+	–	90.2	8.1	1.5	–
Rat	1	63.7	16.6	2.3	17.4	73.5	14.2	–	12.8
	3	58.8	28.0	+	13.2	67.1	22.5	3.0	7.4
	6	46.3	51.8	–	1.8	60.0	39.8	+	–
Controls (Rat)	6	87.7	11.5	+	–	91.6	7.6	0.8	—

Two effects of dilution of the tissue system are of interest 1) A reduction in the rate of degradation of the substrate by a factor of over three, 2) the consistent occurrence of a substantial fraction of total I^{131} in chromatogram peaks described as "unidentified." This fraction was found to consist of more than one iodinated component and will be discussed later. In all cases, the amount of I^{131} in this unknown fraction reached a peak in the early time intervals and decreased very markedly after the first hour of incubation. In experiments with thyroxine, a small amount of I^{131} (never exceeding 5–8% of the total) was found in the peak corresponding to 3:5:3′-triiodothyronine. The occurrence of T_3 was, however, not a regular feature and this compound has been included in the group of unidentified substances mentioned in Table 2.

To determine something of the nature of what is presumed to be one or more enzymes responsible for the degradation of thyroxine and triiodothyronine, certain well-known inhibitors of enzymes and of biological oxidation were studied (23). The results are presented in Table 3.

TABLE 3. DEGRADATION OF L-THYROXINE AND L-3:5:3′-TRIIODOTHYRONINE IN "DILUTE" RAT BRAIN SUSPENSION IN THE PRESENCE OF VARIOUS SUBSTANCES (INCUBATION TIME 6 HRS.)

Substance added	Concn.	*l*-thyroxine, 1.6×10^{-9} *M*				*l*-triiodothyronine, 1.8×10^{-9} *M*			
		% total I^{131} distributed as				% total I^{131} distributed as			
		T_4	I^-	O	Unidentified	T_3	I^-	O	Unidentified
None		48.2	46.6	—	5.2	55.3	37.5	—	6.2
Control		88.9	11.1	—	—	89.9	10.1	—	—
NaCN	10^{-3} *M*	73.7	23.3	—	3.0	66.0	31.0	2.1	+
$NaAsO_2$	10^{-3} *M*	30.2	69.8	–	–	57.3	42.6	–	–
NaF	10^{-2} *M*	66.0	29.0	+	4.6	63.7	29.8	3.0	3.5
Octan-2-01	Saturated Soln.	58.2	22.9	–	8.7	71.6	22.0	+	6.0
Methylene Blue	0.05 % (w/v)	58.2	35.8	2.5	3.5	52.1	47.6	–	+
Janus Green	0.005 % (w/v)	67.6	28.1	+	4.0	56.3	39.3	+	5.1
$HgCl_2$ (before H_2S)	10^{-3} *M*	68.6	6.1	4.0	21.3	75.0	5.2	1.8	18.0
$HgCl_2$ (after H_2S)	10^{-3} *M*	39.2	51.4	3.0	5.3	27.0	63.1	4.1	4.1
H_2S alone	Saturated	41.5	54.7	2.0	1.5	46.1	51.0	–	2.5

Only three substances produced significant inhibitions in the rate and nature of degradation of thyroxine and 3:5:3′-triiodothyronine: NaCN, 2-octanol and $HgCl_2$. The latter substance is of especial interest as it effectively prevented the formation of any inorganic iodide after incubation, while permitting the formation of large amounts of unidentified iodinated compounds. Furthermore, after precipitation of $HgCl_2$ with H_2S and reincubation for six hours, the inhibition was reversed with the formation of the usual amount of iodide and a rapid diminution in the amount of the other iodinated products. $NaAsO_2$ accelerated the rate of breakdown of thyroxine only, but, for both the hormones, iodide was the only iodinated metabolite found after incubation.

Time-Curve Studies and Effect of Substrate Concentration

Although the fraction of total I^{131} in each of the major iodinated compounds has been determined in all cases, only the values of I^{131} in the unchanged thyroxine or 3:5:3′-triiodothyronine were considered for time-curve studies. In earlier experiments the substrate concentration was found to be a major factor determining the results obtained and this problem was also studied further. "Dilute" suspensions were used for all quantitative studies and some typical results on dog, rat and chick brain preparations are presented in Figure 1.

A very similar pattern is observed in the way both thyroxine and 3:5:3′-triiodothyronine are metabolized by the preparations of dog, rat and chick brains. The slope of the degradation curve which is steep for the first 2–3 hours flattens out considerably by the sixth hour, and at lower levels of substrate concentration, its shows a multiphase character. A high degree of sensitivity of the rate of degradation to the amount of substrate present is observed. At doses near the serum level of thyroxine, i.e. 5×10^{-8} *M* to 10^{-7} *M*, a very near total inhibition of the whole system is brought about. In more concentrated brain suspension the threshold for this inhibition due to substrate concentration is raised more or less proportionately. For example, the following result was obtained with rat brain suspensions at 1 gm. tissue/5.0 ml. medium, six hours after incubation with thyroxine:

Substrate Concentration:	5×10^{-8} *M*	10^{-7} *M*	5×10^{-7} *M*	5×10^{-6} *M*
Per cent Total I^{131} as unchanged thyroxine	41.2	62.7	69.5	84.4

In view of the greater biological and chemical instability of 3:5:3′-triiodothyronine (24, 25) it was surprising to note that thyroxine was consistently degraded at a more rapid rate than 3:5:3′-triiodothyronine.

The Nature of Metabolities of l-Thyroxine and l-3:5:3′-Triiodothyronine

Inorganic iodide was the major and most consistent iodinated metabolite of all incubations. The group of iodinated compounds designated so far as "unidentified" was found in all experiments performed with "dilute"

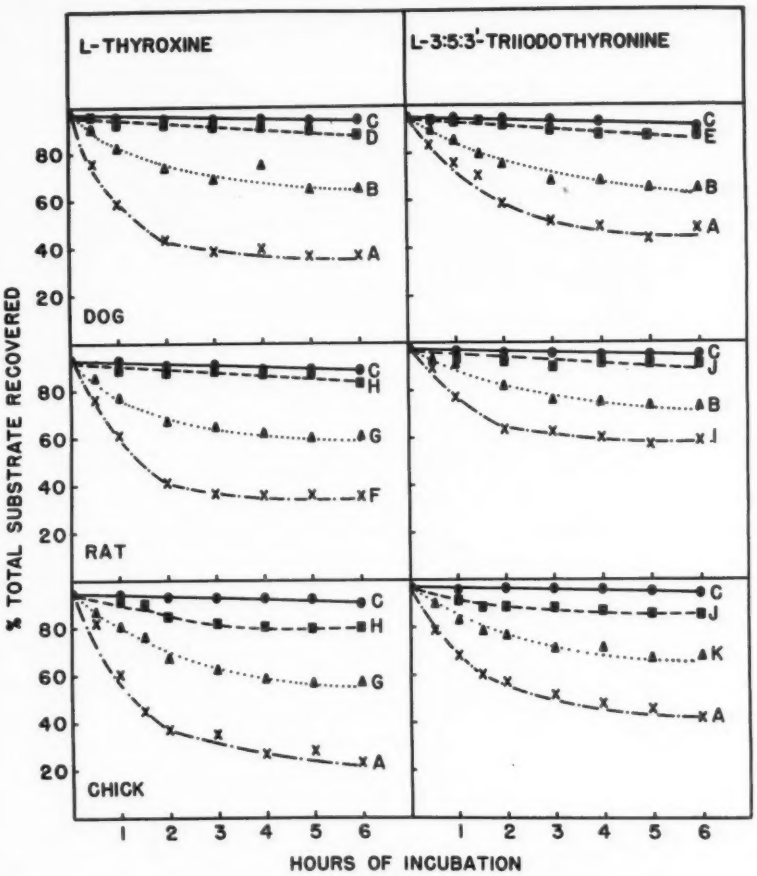


FIG. 1. Degradation of l-thyroxine (T_4) and l-3:5:3′-triiodothyronine (T_3) when incubated with "dilute" dog, rat and chick brain suspensions (1 gm. tissue/15 ml. suspension medium) and the effect of substrate concentration on the rate of degradation, in function of time. Each value represents the average from two independent incubations analyzed by not less than two different solvent systems for chromatography.

Substrate concentrations: A = 5×10^{-9} *M*; B = 2×10^{-8} *M*; D = 1.3×10^{-6} *M*; E = 10^{-6} *M*; F = 3.5×10^{-9} *M*; G = 4×10^{-8} *M*; H = 6×10^{-7} *M*; I = 4×10^{-9} *M*; J = 7×10^{-7} *M*; K = 5×10^{-8} *M*.

C = Control incubations.

systems. Quantitatively, the occurrence of these compounds followed a regular and definite pattern. Attempts made to identify them are described below.

In Figure 2 are reproduced some typical chromatographic recordings of I^{131} distribution in samples of "dilute" brain tissue suspensions after incuba-

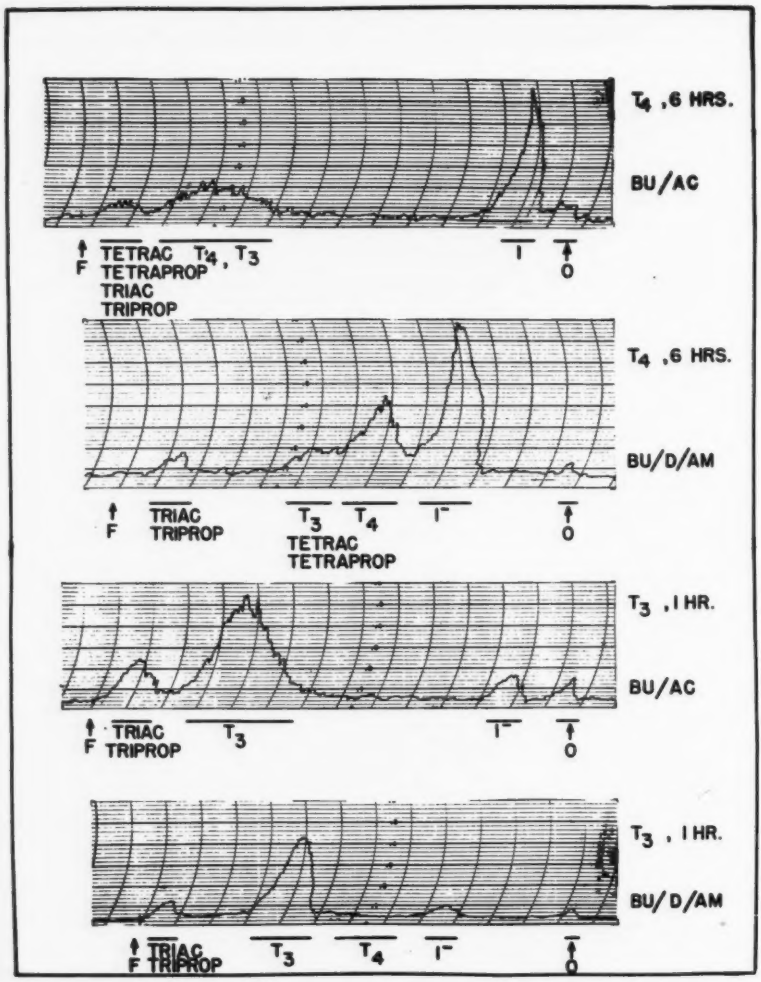


FIG. 2. I^{131} recording of chromatograms of iodinated products of incubation of l-thyroxine (T_4) and l-3:5:3′-triiodothyronine (T_3) with "dilute" rat brain suspension (1 gm. tissue/15 ml. suspension medium).

Solvent systems: Bu/Ac = n-butanol-acetic acid-water.
Bu/D/Am = n-butanol-dioxane-ammonia.
O = Origin of chromatogram.
F = Solvent Front.

tion with thyroxine (for 6 hours) and 3:5:3′-triiodothyronine (for 1 hour). The distribution and homogeneity of the unidentified components depends upon the chromatographic solvent system used.

In order to be satisfied that the different chromatographic peaks were

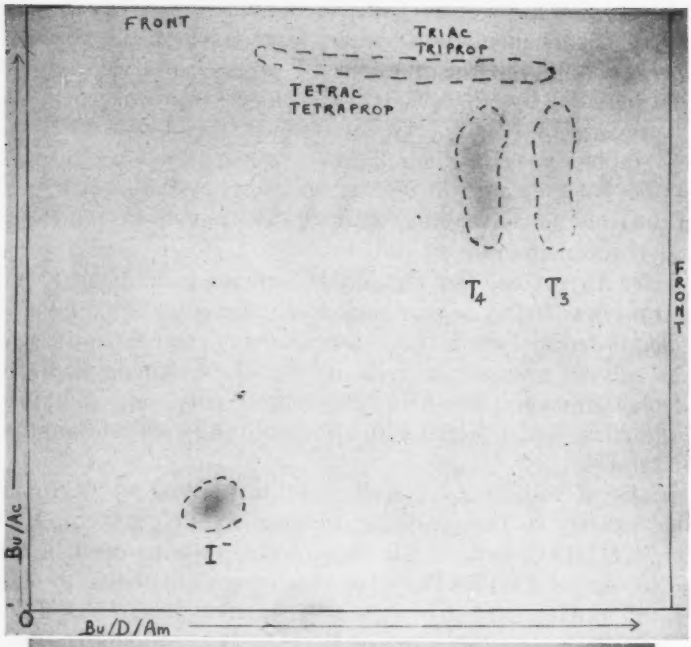
not artefacts, 25 cm.-wide preparative chromatograms were made with 0.2 to 0.4 ml. of each incubation sample. After development, portions of the paper corresponding to the different I^{131} components were eluted with n-butanol followed by 5% NH_4OH. The eluates were concentrated under reduced pressure (5–12 mm. Hg.) at temperatures below 25° C. and re-chromatographed with the four different solvent systems. In each case, not less than 85% of the radioactivity was recovered in a single peak with a Rf, in the same solvent system, that was identical to that in the original preparative chromatogram.

Hence for thyroxine, the non-iodide iodinated component, near the solvent front was found as one peak when the chromatographic solvent system was n-butanol-acetic acid; but this was resolved into two peaks when the solvent system used was n-butanol-dioxane-ammonia, or ter-amyl alcohol-ammonia. For triiodothyronine, only one iodinated peak, outside of iodide, was detected with all the four different chromatographic solvent systems.

The peaks of radioactivity seen after incubation of thyroxine corresponded exactly to the positions occupied by TETRAC and TRIAC. However, TETRAC had, in all the solvent systems used, a mobility identical to that of TETRAPROP so that it was impossible to determine whether the radioiodinated compound in this position was TETRAC, TETRAPROP or a combination of both of them. Similarly it was not possible to be sure of the identity of the other peak since TRIAC and TRIPROP also had identical mobilities. After incubation of triiodothyronine a spot of radioactivity corresponding to TRIAC or TRIPROP was seen. Although TETRAFORM is indistinguishable from TETRAC or TETRAPROP and TRIFORM can not be differentiated from TRIAC or TRIPROP in these solvent systems, the results of electrophoresis described below eliminate these formic acid derivatives.

In bidimensional chromatograms (Figs. 3a and 3b) developed with n-butanol-acetic acid-H_2O and n-butanol-dioxane systems, as the first and second dimensions respectively, the triiodo- and tetraiodo groups of side-chain analogues are not as well resolved as would be expected from results in each of the single dimensions (possibly due to employing larger amounts of the sample than in monodimensional chromatograms). Yet in all cases, the spots on the autograms of bidimensional chromatograms were exactly superimposable on the chemically-stained spots of the carriers added. In the Figures 3a and 3b, the dotted lines represent the tracing of the area occupied by carrier substances added before development of the chromatograms. The faint spot of T_3 observed on the chromatogram of a T_4 incubation (Fig. 3a) was detected in less than half the samples studied whereas the other metabolites were consistently present.

The possibility that the group of iodinated compounds found besides the iodo-thyronines and iodide represent the acetic and propionic acid ana-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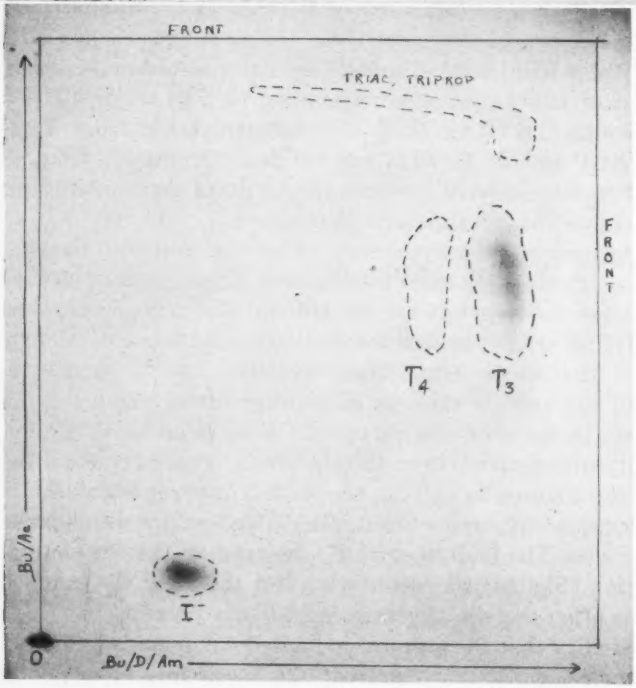
FRONT
TRIAC, TRIPROP
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T_4
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Bu/Ac
I⁻
O
Bu/D/Am

logues of T_4 and T_3, was strengthened by results obtained with paper electrophoretic analysis (Figure 4).

Attempts to detect 3:3′-diiodothyronine were unsuccessful as this compound was found to migrate poorly in the methanol-CH_3COONH_4 system (26) in the presence of brain tissue suspension.

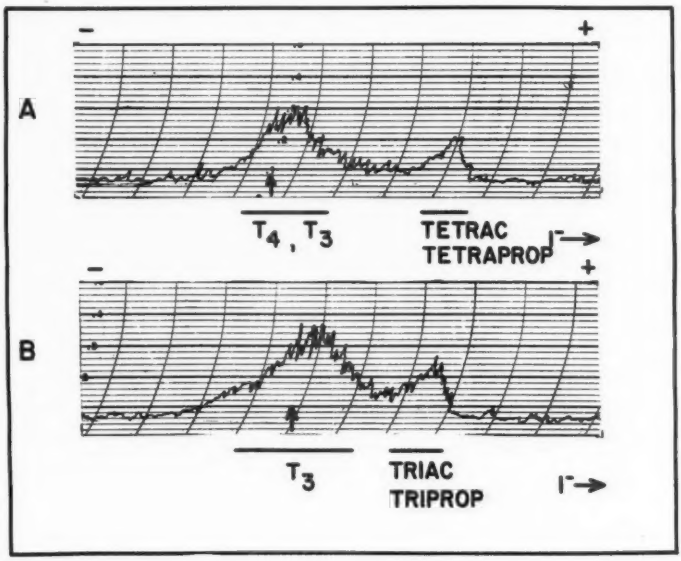

FIG. 4. Paper electrophoresis of iodinated products of incubation of l-thyroxine and l-3:5:3′-triiodothyronine with "dilute" rat brain suspension (1 gm. tissue/15 ml. suspension medium). Electrophoresis with 0.2 *M* $(NH_4)_2CO_3$ buffer at pH 9.1 at 135 v. for six hours. Position of inorganic iodide not shown.

DISCUSSION

The above results show that there are in dog, rat and chick brain, systems, presumably enzymatic, which degrade thyroxine and 3:5:3′-triiodothyronine. The active system in the tissue is thermolabile, being inactivated by heating at 100° for 20–30 minutes and its activity exhibits

FIG. 3. Radioautogram of two dimensional chromatography of iodinated products of incubation of a) l-thyroxine (T_4) and b) l-3:5:3′-triiodothyronine (T_3) with "dilute" rat brain suspension (1 gm. tissue/15 ml. suspension medium). Abbreviations refer to position of added carriers. Dotted lines represent the position of different carriers as revealed by chemical staining.

Solvent systems: Bu/Ac = n-butanol-acetic acid-water.
Bu/D/Am = n-butanol-dioxane-ammonia.
O = Origin of chromatogram.
F = Solvent fronts.

little variation under aerobic or anaerobic conditions. The tissue structure need not be preserved for this activity since cell-free preparations were obtained that possessed properties identical to those observed in brain tissue slices and cell suspensions.[7]

Prior to this work, Sprott and Maclagan (27) had described a system in rat liver, and other tissues (including brain) which was said to deiodinate thyroxine and triiodothyronine. Since this system was barely active at a physiological pH, inhibited by cyanide and anaerobiosis and difficult to inactivate by boiling, it is not readily comparable to the brain system described in the present report. Furthermore, their tissue preparation appeared to be active at substrate concentrations of 20 to 25,000 times the level of thyroxine in blood, whereas the system just described is almost completely inactive at these levels. The difficulty in comparing our results with those of Sprott and Maclagan is increased by the differences in techniques used to determine deiodination or degradation of the hormones. Larson, Tomita and Albright (28) have described an "adaptive thyroxine deiodinating system" in rat kidney slices which converts thyroxine to triiodothyronine. In our experience (see Figure 2) the solvent systems for chromatography used by these workers could not clearly differentiate between T_3, TETRAC and TETRAPROP. More recently, however, Albright, Larson, Tomita and Lardy (29) have reported the formation of TETRAC from thyroxine following incubation with rat kidney mitochondria.

It is important to note in considering the work of Albright *et al.*, and our studies that only the iodine atoms of the phenolic ring of the T_3 and T_4 molecules were labeled with I^{131}. After the loss of these iodine atoms, the molecule can no longer be traced.

From the results described in the present work, the rate of degradation of the two hormones and the nature of the products formed were dependent on both the concentration of the tissue preparation and that of the substrate itself. With the "concentrated" systems (Table 1), only deiodination of thyroxine and triiodothyronine was observed. Dilution of the preparations (Table 2) led to a diminution in the rate of deiodination and also resulted in the appearance of iodinated compounds not observed in more concentrated preparations. These compounds were best seen in shorter incubations. Prolonged incubations (from 6–12 hours) with the "dilute" preparations resulted in inorganic iodide as the major iodinated metabolite. It appears that more than one enzyme systems is involved in the ultimate degradation of thyroxine and 3:5:3′-triiodothyronine to iodide and that dilution of the preparations affects to a different extent the reaction velocities of each system (30). Hence, it is likely that the group of

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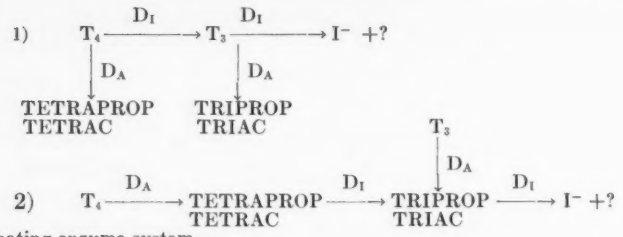
[7] A subsequent communication will deal with the problem of localization and fractionation of the active component in brain tissue.

unidentified compounds were actually formed in the "concentrated" preparations but were too rapidly deiodinated to be observed. Different substrate dose levels used with "dilute" preparations varied from concentrations 10 to 20 times lower to about 1,000 times higher than the circulating level of thyroxine. Since the exact level of 3:5:3′-triiodothyronine in serum is not yet known (31, 32), no comparison is possible. The unexpected slower rate of degradation of triiodothyronine than of thyroxine is difficult to explain. The high degree of sensitivity of the brain preparations to substrate concentration appears to reflect the absence of an efficient detoxication mechanism such as found in the liver and to some extent in the kidney (33, 34).

The unknown iodinated compound observed by Alpers and Rall (15) in human cerebrospinal fluid was not encountered among the incubation products analyzed. On the other hand, what appears to be a series of deaminated derivatives of thyroxine and 3:5:3′-triiodothyronine were characterized. The significance of these derivatives arises from the fact that the pyruvic, propionic, acetic and formic acid side-chain derivatives of thyroxine and 3:5:3′-triiodothyronine have been recently reported to have some properties of the thyroid hormones and, in some biological tests, to possess a higher potency or a shorter time lag preceding their action (35–39). Barker (40), however, has not been able to reproduce some of the biological activity tests on these derivatives. Of these derivatives, the triiodo- and tetraiodo-pyruvic acid analogues have been found in bile and urine but only in significant amounts after the administration of rather large doses of the corresponding amino-acids (41). More recently, Roche, Michel, Jouan and Wolf (42) have identified TRIAC in kidneys of thyroidectomized rats injected with triiodothyronine. It should be pointed out here that in our work it was not possible to distinguish TRIAC from TRIPROP nor TETRAC from TETRAPROP (Figs. 2, 3, and 4). It is not clear, therefore, at what step the side-chain degradation ceases before the molecule loses its iodine atoms in the 3′ and 5′ positions. Thyroxine can also undergo a direct partial deiodination without any side-chain modification to give 3:5:3′-triiodothyronine, although the amount and frequency of occurrence of the latter substance was low. Direct deiodination of 3:5:3′-triiodothyronine to 3:3′-diiodothyronine was not observed in brain although it has been noted by other workers (42) in the rat kidney *in vivo* (along with TRIAC and iodide).

Since mercuric chloride reversibly inhibits only deiodination, it seems quite clear that there are at least two enzyme systems responsible for degradation of thyroxine and triiodothyronine. Two general schemes of degradation of thyroxine and triiodothyronine (or a combination of these) appear to be possible, dependent upon the relative affinities of the deiodinating and deaminating enzymes for the compounds involved. These are:



D_I = deiodinating enzyme system.
D_A = deaminating enzyme system.

Scheme 1 does not appear to be probable in our "dilute" brain tissue preparations because very little if any triiodothyronine is found while the deaminated derivatives are formed in significant amounts, particularly if D_I is blocked by $HgCl_2$. Therefore, the more likely pathway seems to be scheme 2 in which the major substrates for the deiodinating enzyme would be the deaminated compounds.

No evidence is as yet available as to whether the transformation of l-thyroxine and l-3:5:3′-triiodothyronine observed *in vitro*, are reproduced in the central nervous system of intact animals, although work has been begun on this problem. It is possible that at the cellular level the thyroid hormones undergo a series of chemical transformations in exerting their biological action and therefore the identification of their metabolites reflect, in part, a mode of action. Although this is a tempting conclusion, this work only opens the question of whether the transformations here observed are designed to provide the central nervous system with fast-acting biologically active metabolites of the thyroid hormones or whether they just represent a chain of metabolic reactions commonly undergone by aromatic amino acids (42).

SUMMARY

Rat, chick and dog brain tissue possesses systems, presumably enzymatic, capable of deaminating and deiodinating thyroxine and 3:5:3′-triiodothyronine. These systems are active at a physiological pH (pH 7.45) and their activity is highly sensitive to changes in both the concentration of substrate and that of the tissue preparation itself. These systems are unaffected by cyanide or anaerobiosis but are inactivated by heating to 100° C. Mercuric chloride reversibly inhibits the deiodinating enzyme while the deaminating enzyme is unaffected. Under these conditions or with dilute preparations of the brain tissue, the propionic and/or acetic acid analogues of both thyroxine and triiodothyronine are formed. It was not possible to distinguish TETRAC from TETRAPROP and similarly TRIAC from TRIPROP but one or more of these were formed. The major product of deiodination was iodide but in some cases a small amount of triiodothyronine was formed as a product of incubation of thyroxine.

Acknowledgment

The authors are highly indebted to Dr. William L. Money for his invaluable assistance and helpful suggestions for this work.

Thanks are also due to Miss Margaret Mazzucco for excellent technical cooperation.

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HYDROCORTISONE SUPPRESSION OF STRESS-INDUCED ADRENAL 17-HYDROXYCORTICOSTEROID SECRETION IN DOGS[1]

JOHN B. RICHARDS[2] AND RALPH L. PRUITT

Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland

THE administration of adrenocortical hormones to intact rats has been shown to inhibit pituitary secretion of ACTH (1–4). Sydnor has recently reported that the increase in blood ACTH which occurs in adrenalectomized rats subjected to ether anesthesia is suppressed by the administration of large doses of hydrocortisone (5). In these previous investigations, ACTH activity was determined by alterations in either the weight or ascorbic acid concentration of adrenal glands of rats.

Exposure to 20% CO_2 (6) and the surgical trauma of adrenal vein cannulation (7) are two stimuli independently capable of producing increased pituitary-adrenal activity in pentobarbital-anesthetized dogs. In the present investigation the effect of these two stimuli upon the adrenocortical response in dogs with increased plasma concentrations of 17-hydroxycorticosteroids was studied. Adrenocortical function was determined by measuring 17-hydroxycorticosteroids in adrenal venous blood. This technique provides a direct and specific index of the secretory activity of the adrenal cortex.

METHODS

Thirty-six male mongrel dogs ranging in weight from 10 to 17 kg. were used in these studies. Twenty dogs (Group I) were employed in the operative trauma studies, and 16 dogs (Group II) in the CO_2 exposure experiments.

The animals in Group I were anesthetized with pentobarbital sodium and the right lumboardrenal veins cannulated according to a procedure described by Hume and Nelson (7). This preparation permits either continuous or intermittent collection of the total venous effluent of the right adrenal gland. Immediately upon cannulation of the lumboadrenal vein, and with the peritoneal cavity stretched open with Balfour retractors, 3 to 5 adrenal venous blood samples were collected intermittently during a 10–15 min. period. To determine maximal adrenocortical secretory activity, ACTH (Corticotropin, Armour, 20 I.U.) was then infused into either a jugular or foreleg vein and adrenal venous blood samples obtained 5 and 10 min. following injection. One half of this group of dogs (nos. 11–20) received a constant intravenous infusion of hydrocortisone (Hydrocortone, Sharpe & Dohme) for one hour prior to and during the operative procedure. The hormone

Received July 20, 1956.

[1] The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or as reflecting the view of the Navy Department or the naval service at large.

[2] Present address: II & IV Medical Services (Harvard), Boston City Hospital, Boston, Mass.

was dissolved in 0.9% sodium chloride (100 mg./1000 ml.) and infused at a rate of 1.0–1.25 mg./kg. body wt./hr. In order to measure the concentration of hormone in the peripheral blood of these animals, a carotid artery was cannulated just before beginning the adrenal vein cannulation procedure (after 1 hr. of hormone infusion).

Twenty-five ml. of arterial blood were collected at the start and finish of the adrenal surgery. Control samples were obtained on the following day. These samples were collected in heparinized tubes and 10 ml. of plasma from each was separated and saved for 17-hydroxycorticosteroid analysis.

The 16 dogs in Group II were anesthetized with pentobarbital sodium and polyethylene cannulas inserted into the right lumboadrenal vein and carotid artery of each animal. The dogs were allowed to recover during a period of 24 hrs. They were then reanesthetized with pentobarbital sodium and placed in a large sealed chamber. Following a control period of 30 min., during which arterial and adrenal venous blood samples were collected, the CO_2 concentration within the chamber was rapidly (3–5 min.) increased to 20%. The oxygen concentration in the chamber was maintained at 21%, the temperature at 24°–25° C, and at all times the chamber was kept at atmospheric pressure. Oxygen concentration within the chamber was measured by continuous sampling through a Beckman oxygen analyzer, model F-3. A Liston Becker CO_2 analyzer, model 16, was used to monitor the chamber atmosphere and activate a system for maintaining the CO_2 concentration at 20%. Adrenal venous blood samples were collected at 15 min. intervals and arterial blood samples at 30 min. intervals for a period of one hour, after which the chamber atmosphere was rapidly changed and the dogs allowed to breathe room air for a period of 1½ to 2 hrs. One half of this group (Dogs 29–36) then received an intravenous infusion of hydrocortisone (100 mg./1000 ml. 0.9% sodium chloride, 1.0–1.25 mg./kg./hr.) and the other half (Dogs 21–28) 0.9% sodium chloride for one hour prior to and during a second one-hour period of exposure to 20% CO_2. The rate of fluid infusion in all animals was 10–12 ml./kg./hr. Arterial and adrenal blood samples were collected in the same manner as those obtained during the first CO_2 period. In addition, 25 ml. of carotid artery blood were collected prior to the fluid infusion, and at the beginning and end of the second CO_2 period. Ten ml. of plasma were separated from each sample and saved for steroid analysis.

The arterial bloods obtained for determination of CO_2 content and pH were collected under anaerobic conditions. Plasma from each sample was analyzed for CO_2 content in the Van Slyke manometric apparatus. Plasma pH was determined with a Beckman pH meter, model G. All adrenal blood samples were obtained by collecting the total venous effluent of the right adrenal gland for a period of either 30 or 60 seconds and were analyzed for 17-hydroxycorticosteroids by the method of Nelson and Samuels (8) as modified by Nelson and Hume (9).

Frequent temperature readings were obtained from indwelling thermometers inserted 6 inches into the rectum of each dog. The range of rectal temperature in these 36 dogs, during the periods of experimentation, was from 37°–39° C.

RESULTS

A. *Operative Trauma*

Table 1 lists the adrenal 17-hydroxycorticosteroid output values in 20 dogs during a period of surgical trauma associated with adrenal vein cannulation. In the control animals (nos. 1–10), maximal adrenal corticoid secretion existed at the time of adrenal vein cannulation as evidenced by the finding that no additional increase in corticoid output could be evoked by the administration of 20 I.U. ACTH (a dose which produces maximal

TABLE 1. SUPPRESSIVE EFFECT OF HYDROCORTISONE ON ADRENAL 17-HYDROXYCORTICOSTEROID SECRETION DURING OPERATIVE TRAUMA IN DOGS

Control dogs			Dogs receiving hydrocortisone infusion					
	Adrenal 17-OHCS secretion μg./min.			Adrenal 17-OHCS secretion μg./min.		Plasma 17-OHCS conc. μg./100 ml.		
Dog No.	At time of adrenal vein cannulation*	Following ACTH injection 20 I.U.†	Dog No.	At time of adrenal vein cannulation*	Following ACTH injection 20 I.U.†	Control	Start of operation	End of operation
1	8.5±1.3‡	9.0	11	1.9±0.3‡	11.0	3	31	37
2	13.9±2.0	13.5	12	2.4±1.4	10.5	1	27	49
3	6.3±0.4	6.1	13	1.2±0.7	11.1	4	36	41
4	11.0±0.5	10.2	14	5.3±1.1	9.6	2	59	92
5	11.0±1.7	10.6	15	2.6±0.5	5.2	1	36	39
6	11.8±0.9	13.8	16	0.9±0.5	12.8	3	30	70
7	11.1±0.5	11.2	17	4.1±2.2	12.6	6	33	48
8	18.1±1.4	19.0	18	2.3±0.6	7.8	4	49	49
9	11.4±0.7	13.0	19	2.5±0.8	15.7	12	52	46
10	9.1±0.5	9.7	20	6.2±1.3	16.6	6	41	62

* Average value of 3–5 samples collected during 15 min. period.
† Average of samples collected 5 and 10 min. after ACTH injection.
‡ Standard deviation.

adrenocortical stimulation). In the animals which received hydrocortisone for one hour prior to and during the operative procedure (nos. 11–20), maximal secretory activity of the adrenal cortex was not present at the time of adrenal vein cannulation. In these animals a marked (2-to-14 fold) increase in adrenal 17-hydroxycorticosteroid secretion was elicited by the intravenous administration of 20 I.U. ACTH. The operative procdeure used in both groups of dogs was identical and all adrenal venous blood samples were collected while the peritoneal cavity was stretched open with Balfour retractors.

B. *Exposure to 20% CO_2*

Table 2 contains the results obtained in 8 control dogs exposed to 20% CO_2 for two separate one-hour periods. Sodium chloride, 0.9%, was infused intravenously for one hour prior to and during the second CO_2 exposure period. An appreciable (3-to-16 fold) increase in adrenal corticoid secretion occurred in all dogs during both periods of CO_2 exposure. The

TABLE 2. CONTROL CO_2 EXPERIMENTS

Dog No.	First CO_2 exposure period				Second CO_2 period—saline infusion (0.9% sodium chloride)						
	Adrenal 17-hydroxycorticosteroid secretions				Adrenal 17-hydroxycorticosteroid secretion				Plasma conc. (μg./100 ml.) 17-hydroxycorticosteroids		
	Controls		20% CO_2—1 hr.		Controls		20% CO_2—1 hr.		Control	Start of CO_2 exposure	End of CO_2 exposure
	No. samples	Avg. μg./min.	No. samples	Avg. μg./min.*	No. samples	Avg. μg./min.	No. Samples	Avg. μg./min.*			
21	4	3.1±1.5†	5	10.6±0.9†	6	4.0±1.2†	5	15.4±2.1†	13	14	20
22	5	3.2±1.5	5	10.5±2.5	6	2.4±1.0	5	13.4±1.8	18	17	19
23	6	1.9±0.4	4	8.0±0.7	4	0.9±0.3	3	6.9±1.0	9	11	12
24	6	2.3±1.5	4	12.9±1.1	6	1.1±0.3	5	15.3±3.0	11	6	12
25	6	2.1±0.7	5	9.6±1.5	6	1.0±0.6	4	9.5±2.1	5	4	5
26	4	2.1±0.6	5	11.8±2.1	6	1.4±0.5	5	8.0±2.2	9	10	2
27	4	1.6±0.9	5	7.3±1.0	6	0.8±0.5	5	6.0±0.8	6	2	5
28	6	0.9±0.2	5	14.2±2.1	6	1.5±0.3	2	11.4±2.2	10	5	7

* Average of values obtained during last 45 min. of 60 min. CO_2 exposure period. Adrenocortical stimulation usually does not occur during first 15 min. of exposure.
† Standard deviation.

adrenocortical response in individual animals was of a similar magnitude during both exposure periods. Likewise, the degree of respiratory acidosis which occurred during the first period of CO_2 exposure (avg. arterial pH 6.94, avg. arterial pCO_2 130 mm. Hg) was the same as that attained during the second exposure period (avg. pH 6.92, avg. pCO_2 132 mm. Hg). Despite the marked increase in adrenocortical secretory activity, only one animal (no. 21) gave evidence of an increase in peripheral plasma 17-hydroxycorticosteroid concentration during CO_2 exposure.

A second group of 8 dogs (Table 3) received hydrocortisone intravenously for one hour prior to and during the second period of CO_2 exposure.

TABLE 3. HYDROCORTISONE SUPPRESSION OF ADRENOCORTICAL STIMULATION IN DOGS EXPOSED TO 20% CO_2

Dog No.	First CO_2 exposure period				Second CO_2 period—with hydrocortisone infusion						
	Adrenal 17-hydroxycorticosteroid secretion				Adrenal 17-hydroxycorticosteroid secretion				Plasma conc. (μg./100 ml.) 17-hydroxycorticosteroids		
	Controls		20% CO_2—1 hr.		Controls		20% CO_2—1 hr.				
	No. samples	Avg. μg./min.	No. samples	Avg. μg./min.*	No. samples	Avg. μg./min.	No. samples	Avg. μg./min.*	Control	Start of CO_2 exposure	End of CO_2 exposure
29	6	1.4±0.5†	5	12.3±2.9†	6	1.6±0.2†	6	2.2±0.7†	8	48	51
30	6	0.9±0.4	4	7.1±2.0	6	1.3±0.5	6	1.6±1.1	5	36	41
31	5	1.5±0.7	4	6.6±2.4	6	1.2±0.4	6	1.8±0.6	13	38	70
32	5	0.6±0.4	4	13.2±2.0	6	1.1±0.3	5	5.2±2.1	1	35	121
33	5	1.4±0.7	5	10.6±1.9	5	1.7±1.4	6	1.5±0.9	11	41	60
34	6	1.4±0.9	5	8.8±1.4	6	0.8±0.6	6	0.6±0.4	6	30	24
35	6	2.3±0.8	4	8.7±2.3	6	2.0±1.3	6	2.4±1.1	6	26	43
36	6	0.8±0.3	4	9.9±0.6	6	1.2±0.9	6	1.0±0.3	8	57	60

* Average of values obtained during last 45 min. of 60 min. CO_2 exposure period. Adrenocortical stimulation usually does not occur during first 15 min. of exposure.
† Standard deviation.

During the initial CO_2 period, all dogs displayed an adrenocortical response and respiratory acidosis (avg. arterial pH 6.90, avg. pCO_2 140 mm. Hg). In the second exposure period, during hydrocortisone infusion, 7 of 8 dogs failed to show an appreciable increase in adrenal corticoid output over control levels, despite the presence of severe respiratory acidosis (avg. arterial pH 6.87, avg. pCO_2 146 mm. Hg). In animal no. 32, an increase in corticoid secretion did occur. This apparent increase may be due to the unusually high concentration of corticoids in the peripheral blood (121 μg./100 ml. plasma at end of CO_2 period) and the volume of adrenal venous blood flow (4 ml./min.).

Adrenal venous blood flow in the dogs exposed to CO_2 (nos. 21–36) ranged between 1.1–4.0 ml./min., and in each case, an increase (10–40%) in blood flow occurred during exposure to 20% CO_2.

DISCUSSION

The results indicate that the surgical trauma associated with adrenal vein cannulation, as performed in these studies, stimulates the adrenal cortex to maximal secretory activity. Previous studies have shown that exposure to 20% CO_2 likewise evokes a maximal adrenocortical stimulation

in anesthetized dogs (6). An increase in plasma concentration of 17-hydroxycorticosteroids from an average control level of 7 (1–18) μg./100 ml. to 48 (26–121) μg./100 ml. suppressed the adrenocortical response in anesthetized dogs subjected either to 20% CO_2 or to operative trauma.

Adrenocortical responsiveness to ACTH is unaltered in the presence of high concentrations of cortical hormones in body fluids (10), and hypophysectomy abolishes the stimulation of the adrenal cortex in dogs exposed to 20% CO_2 or subjected to surgical trauma (6). It is thus apparent that in the present experiments the suppressive action of the administered hydrocortisone was upon the release of ACTH from the adenohypophysis. A direct effect upon hypothalamic centers concerned with regulation of pituitary ACTH secretion cannot be ruled out. McCann and Sydnor have reported that suitably placed hypothalamic lesions prevent the rise in blood ACTH concentration which occurs in adrenalectomized rats subjected to ether anesthesia and hemorrhage (11). If the increased secretion of pituitary ACTH in anesthetized dogs subjected to either 20% CO_2 or to operative trauma is mediated through the hypothalamus, it would appear that hypthalamic stimulation (in the hydrocortisone experiments) was either absent or incapable of overcoming the suppressive effect of increased concentrations of 17-hydroxycorticosteroids in the blood stream. However, by employing the same techniques as those used in the present studies, it was found that hydrocortisone administration did not suppress the pituitary-adrenocortical response to chlorpromazine administration (12). These findings support the contention of Sayers (13), that the inhibitory effect of cortical steroids upon stress-induced pituitary ACTH secretion is dependent upon the type and severity of stress as well as the method and amount of hormone administration.

SUMMARY

Maximal adrenal 17-hydroxycorticosteroid secretion occurs in pentobarbital anesthetized dogs exposed to 20% CO_2 or subjected to the surgical trauma of adrenal vein cannulation. The adrenocortical stimulation, in both cases, was suppressed by the constant intravenous infusion of hydrocortisone for one hour prior to and during the period of stress.

Acknowledgment

The authors gratefully acknowledge the valuable technical assistance of P. J. Clauser, M. MacKechnie, G. P. Hanlon, S. E. Taylor, A. V. Budris, D. W. Raw, and G. W. Baldauf.

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THE EFFECT OF FURADROXYL TREATMENT AND X-IRRADIATION ON THE HYALURONIDASE CONCENTRATION OF RAT TESTES[1]

E. STEINBERGER AND WARREN O. NELSON[2]

Department of Anatomy, State University of Iowa, Iowa City, Iowa

RIISFELDT (1, 2), on the basis of studies of hyaluronidase concentration in testes of rats under various experimental conditions, suggested that the spermatocytes are the testicular cells responsible for hyaluronidase production. Steinberger and Nelson (3), analyzing the effects of hypophysectomy, cryptorchidism, and estrogen and androgen treatment on the hyaluronidase concentration and morphological picture of rat testes, secured evidence that cells beyond the stage of spermatocytes, the spermatids, are more likely to be the site of hyaluronidase production than are the spermatocytes.

The present study was conducted to provide additional evidence concerning the cell of origin of hyaluronidase in the rate testis.

MATERIALS AND METHODS

In this study 179 adult male rats of the Long-Evans strain were used. The animals were divided into four groups. One group was treated with furadroxyl[3] (3 gm. furadroxyl in 1000 gm. of diet) for periods ranging from 5 to 30 days. The second group of animals was treated with the same dose of furadroxyl for 30 days and then allowed to recover for periods ranging from 20 to 60 days. The third group was treated with the same dose of furadroxyl, plus 50 μg. of estradiol-17β daily for 30 days and then allowed to recover for periods ranging from 15 to 40 days. The fourth group received a single dose of x-rays, 600 r, to the scrotal area. This dose had been shown in other studies to cause permanent sterility in the large majority of rats by destroying the spermatogonia. During the irradiation procedure the body of the animal, except for the scrotum and hind legs, was shielded with heavy lead plates. The animals were sacrificed at periods ranging from 5 to 35 days after irradiation.

Testicular tissue taken for histological studies was fixed in Bouin's solution, embedded in paraffin and cut at 6 μ. Sections were stained with the Masson or Gomori trichrome techniques, or with periodic acid-Schiff's stain and a hematoxylin counterstain.

The technique of hyaluronidase extraction from the testes and the assay procedure used for the quantitation of the enzyme have been described in our previous publication (3). In the present paper, as in the earlier study, hyaluronidase concentrations are expressed in Schering Turbidity Reducing units.

Received July 25, 1956.

[1] This work has been aided by grant RG-1778 from the National Institutes of Health, United States Public Health Service.

[2] Present address: Population Council, Rockefeller Institute, New York City.

[3] We should like to thank the Eaton Laboratories, Norwich, N. Y. for their generosity in providing supplies of furadroxyl for this investigation.

EXPERIMENTAL

Furadroxyl Series

a. Effects of furadroxyl treatment. Table 1 reveals that the earliest significant decrease in testicular hyaluronidase concentration occurred on the 10th day of treatment, concomitantly with the initial drop in testicular weight. On the 15th day only a negligible amount of hyaluronidase could be detected, and the testicular weights had continued to decrease. No further decline in testicular weight was observed on the 20th day; however the testes of rats treated for 30 days were distinctly smaller. In both of the latter groups no hyaluronidase was detected.

Definite histological changes were observed on the 10th day of treatment. These coincided with the initial decrease in hyaluronidase concentration and the initial drop in testicular weight. Many tubules revealed

TABLE 1. EFFECT OF FURADROXYL TREATMENT ON HYALURONIDASE CONCENTRATION OF RAT TESTES

Days treated	No. of rats	No. of assays	Body wt., gm.	Testicular wt., mg.	Hyaluronidase content, units per gm.
5	8	5	306 ± 12.2*	3202 ± 87	94.0 ± 4.0
8	4	8	340 ± 23.1	3050 ± 92	78.0 ± 5.2
10	8	7	320 ± 21.5	2437 ± 176	37.0 ± 6.2
15	8	4	300 ± 18.3	1603 ± 68	1.2 ± 0.63
20	4	2	311 ± 28.1	1743 ± 100	0.0
30	8	4	325 ± 31.2	1152 ± 102	0.0
Normal controls	35	18	391 ± 37	3150 ± 165	87.2 ± 5

* Mean ± Standard error of the mean.

early evidence of spermatogenic arrest at the stage of primary spermatocytes, characterized by increased numbers of these cells and absence of young spermatids. On the other hand, some tubules continued to show an apparently normal spermatogenic process (Fig. 1). On the 15th day of treatment a picture of spermatogenic arrest prevailed in most of the tubules (Fig. 2). These tubules contained Sertoli cells, spermatogonia and dividing primary spermatocytes, but virtually no elements beyond spermatocytes except for occasional degenerating forms of spermatids and fragmented sperm. Testes of animals sacrificed on the 20th and 30th day of treatment revealed a picture of spermatocytic arrest with numerous primary spermatocytes, but no elements beyond this stage of maturation. The Leydig cells remained morphologically intact and abundant in all instances, and the accessory reproductive organs showed no evidence of interference with androgen secretion.

b. Recovery following furadroxyl treatment. Small quantities of hyaluronidase could be detected in testes of rats sacrificed on the 25th day of recovery. Thereafter the concentration of hyaluronidase increased progressively (Table 2), but not until the 35th and 40th day had the enzyme con-

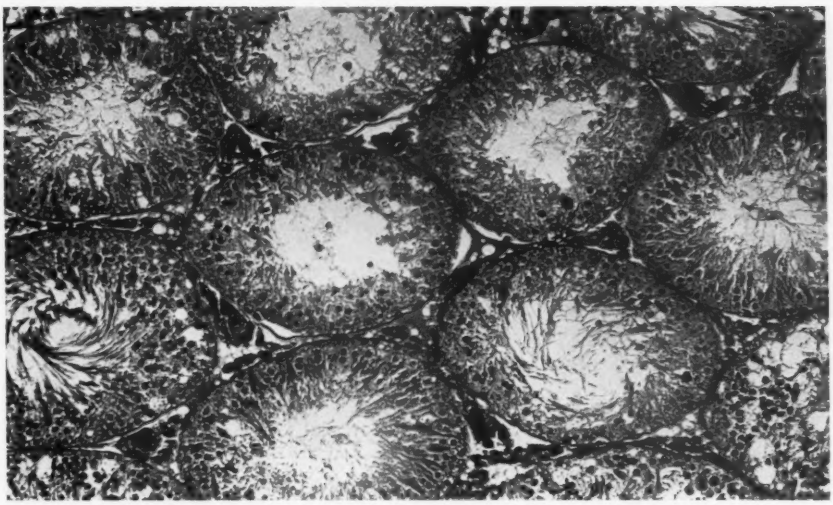

FIG. 1. Testis of rat treated with furadroxyl for 10 days. ×175.

centration become substantial, and only on the 60th day did it approach normal levels. Aside from the early recovery period (25th, 30th, and 35th days) where the weights showed no significant change (although the enzyme had begun to reappear in gradually increasing quantities), the rate of recovery of testicular weights was essentially parallel with the increase in enzyme concentration.

Microscopic evidence suggested resumption of spermatogenesis on the 20th day of recovery. At this time most of the tubules contained only

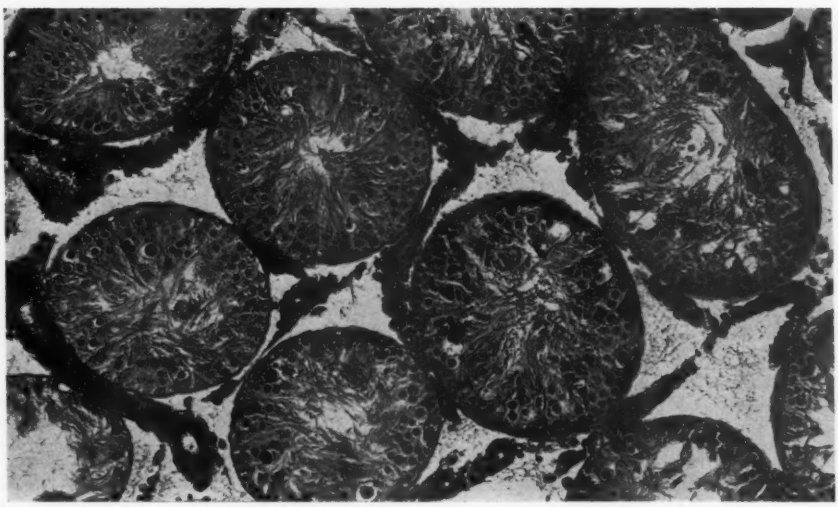

FIG. 2. Testis of rat treated with furadroxyl for 15 days. ×175.

TABLE 2. HYALURONIDASE CONCENTRATION IN RAT TESTES AFTER RECOVERY FOLLOWING FURADROXYL, AND FURADROXYL AND ESTROGEN TREATMENT

Period of recovery days	No. of rats	No. of assays	Body wt., gm.	Testicular wt., mg.	Hyaluronidase content units per gm.
			Furadroxyl		
20	4	2	325 ± 26*	1450 ± 83	0.0
25	8	7	321 ± 16	1331 ± 68	12.0 ± 0.63
30	4	3	322 ± 12	1265 ± 71	13.0 ± 0.71
35	6	6	360 ± 15	1520 ± 20	37.2 ± 2.9
40	7	10	373 ± 21	1861 ± 61	47.8 ± 4.1
60	8	4	385 ± 18	2120 ± 74	71.2 ± 3.9
			Furadroxyl and estrogen		
15	4	3	341 ± 12	1507 ± 178	Trace
20	4	4	356 ± 8	1640 ± 112	20.8 ± 2.3
30	4	3	340 ± 13	2406 ± 182	54.3 ± 2.5
40	4	3	371 ± 15	2760 ± 196	76.3 ± 2.0

* Mean ± Standard error of the mean.

Sertoli cells, spermatogonia, and primary spermatocytes, but an occasional tubule showed normally dividing primary spermatocytes, secondary spermatocytes and spermatids in an early stage of development—the Golgi phase. Larger numbers of young spermatids could be observed on the 25th day of recovery, some of these being in the cap phase of development (Fig. 3). On the 30th day of recovery, the picture had not changed remarkably, except that more tubules contained spermatids in the Golgi phase with an occasional tubule showing spermatids in the maturation phase. After 35 days the great majority of tubules contained spermatids in various stages of development, with the Golgi phase and the cap phase

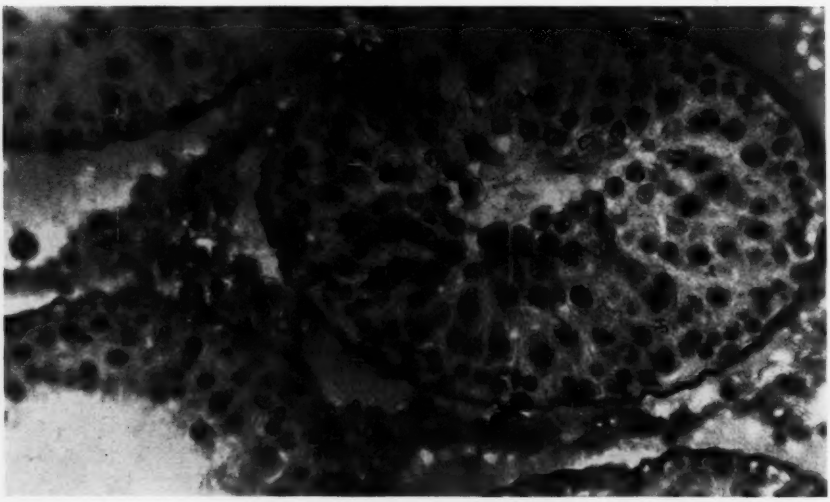

FIG. 3. Testis of rat on the 25th day of recovery after treatment with furadroxyl for 30 days. ×275.

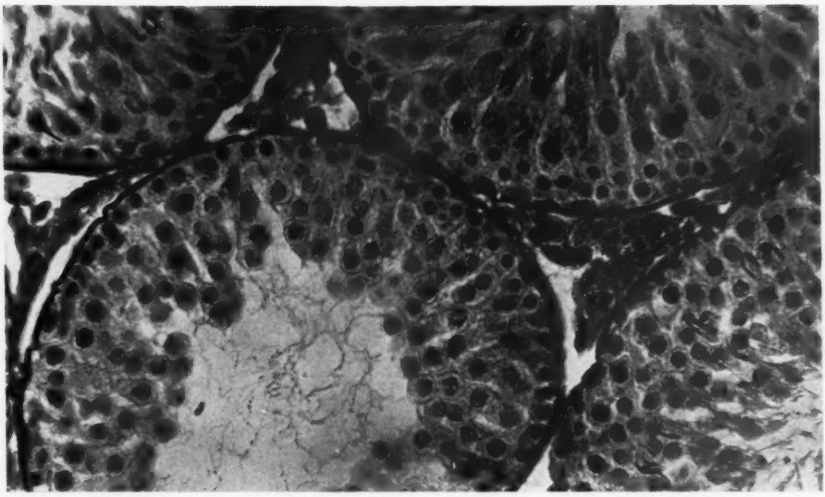

FIG. 4. Testis of rat on the 35th day of recovery after treatment with furadroxyl for 30 days. ×275.

predominating. An appreciable number of tubules, however, did contain spermatids in all stages of the maturation process (Fig. 4). No further change could be noted on the 40th day, aside from an increased number of tubules in which the spermatids had reached the latest stages of maturation. An occasional tubule was found which had not yet progressed beyond the early stages of recovery. All stages of spermatogenesis could be detected

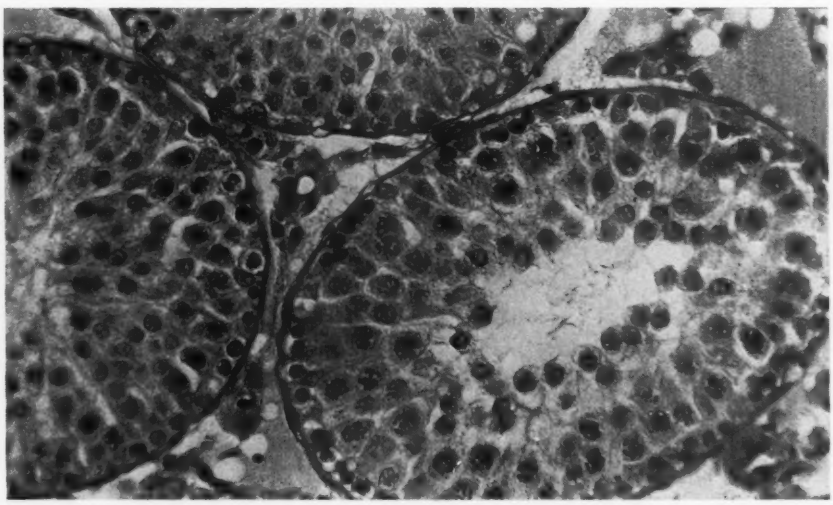

FIG. 5. Testis of rat on the 20th day of recovery after treatment with furadroxyl and estradiol for 30 days. ×275.

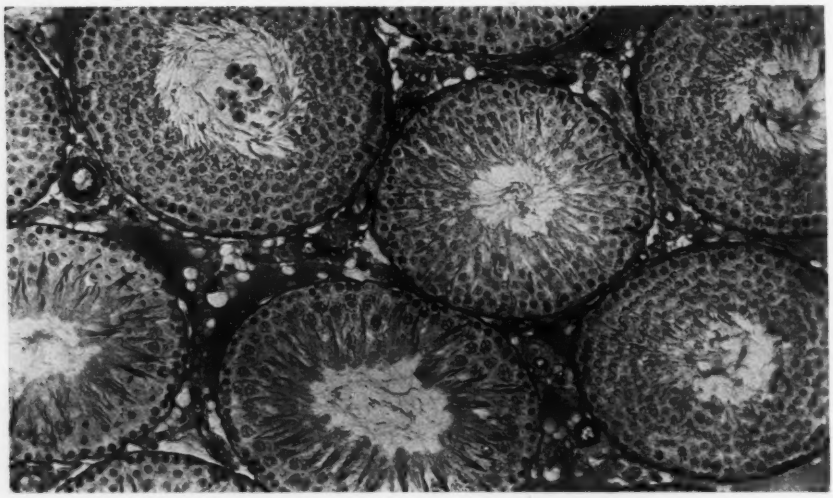

FIG. 6. Testis of rat on the 35th day of recovery after treatment with furadroxyl and estradiol for 30 days. ×275.

on the 60th day in the vast majority of tubules, with many containing large numbers of mature sperm.

c. Recovery following furadroxyl-estrogen treatment. As early as the 15th day of recovery, traces of hyaluronidase could be detected, and by the 20th day the enzyme concentration had reached about 20 per cent of the normal value. From this point the increase was rapid, and by the 40th day approached concentration observed in normal testes (Table 2).

The histological picture of testes from rats sacrificed on the 15th day of recovery revealed a significant number of tubules with spermatids in the early stages of development, chiefly the Golgi phase and occasionally the cap phase, but in most of the tubules spermatogenesis had not proceeded beyond the stage of primary spermatocytes. On the 20th day, the majority of tubules contained spermatids, many of which were in the cap phase and some in the maturation phases (Fig. 5); while on the 30th day

TABLE 3. EFFECT OF X-IRRADIATION ON HYALURONIDASE CONCENTRATION OF RAT TESTES

Days after x-irradiation	No. of rats	No. of assays	Body wt., gm.	Testicular wt., mg.	Hyaluronidase content, units per gm.
5	8	4	315 ± 19*	2880 ± 129	96.0 ± 4.8
10	8	4	320 ± 15	2675 ± 118	116.0 ± 6.2
15	12	8	347 ± 15	2517 ± 71	101.0 ± 12.0
20	12	11	345 ± 9	2042 ± 63	61.4 ± 10.8
25	7	6	360 ± 12	2237 ± 39	56.4 ± 3.5
35	7	6	385 ± 16	1578 ± 53	4.2 ± 0.37
Normal controls	35	18	391 ± 37	3150 ± 165	87.2 ± 5.0

* Mean ± Standard error of the mean.

advanced stages of spermatid development and even mature sperm were present in a large percentage of the tubules. By the 35th to 40th day essentially normal spermatogenesis had been restored throughout the testes, (Fig. 6).

X-Irradiation Series

Examination of the data given in Table 3 shows that five days after irradiation the testicular weights remained within normal limits, while the hyaluronidase concentration was slightly, but probably not significantly, above normal levels. Ten days after irradiation, however, the hyaluronidase concentration was well above the normal value, and the testicular weights had decreased slightly below the normal average. Fifteen days after irradiation the testicular weights were definitely diminished, whereas the hyaluronidase concentration, though lower than on the 10th day, con-

FIG. 7. Testis from normal rat. Note particularly the layer of spermatogonia resting on the basement membrane of each of the tubules. ×500.

tinued to be above the normal value. The earliest significant decrease in concentration of enzyme was noted 20 days after irradiation, and was associated with a further drop in testicular weights. From this point on, the hyaluronidase concentration declined progressively, and on the 35th day after irradiation became negligible.

Concomitantly with the increase in hyaluronidase concentration 10 days after irradiation, definite microscopic changes were observed and should be compared with the histologic condition of the normal testis (Fig. 7).

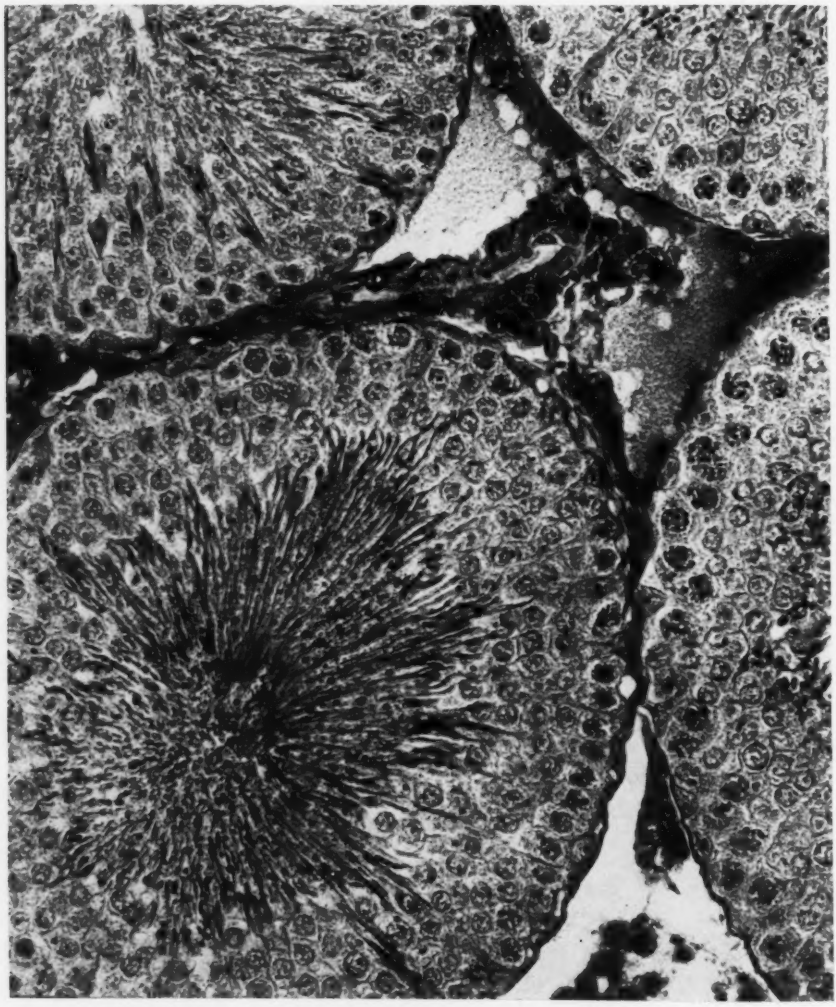

FIG. 8. Testis from rat 10 days after x-irradiation of the scrotal area. Note the absence of spermatogonia. The cells resting on the basement membrane are primary spermatocytes and occasional Sertoli cells. ×500.

FIG. 9. Testis from rat 15 days after x-irradiation of the scrotal area. Note that primary spermatocytes as well as spermatogonia are now absent. The cells resting on the basement membrane are spermatids and occasional Sertoli cells. ×500.

Throughout the x-irradiated testes the tubules showed complete absence of spermatogonia, but spermatocytes, spermatids, and sperm were intact and histologically normal (Fig. 8). A striking picture was observed in the testes of animals sacrificed 15 days after irradiation, spermatogonia being completely absent and most tubules lacking spermatocytes. The young spermatids in the latter case were adjacent to the basement membrane of the tubules and the more mature forms closer to the lumen. These spermatids were normal in appearance, and together with a large number of intact

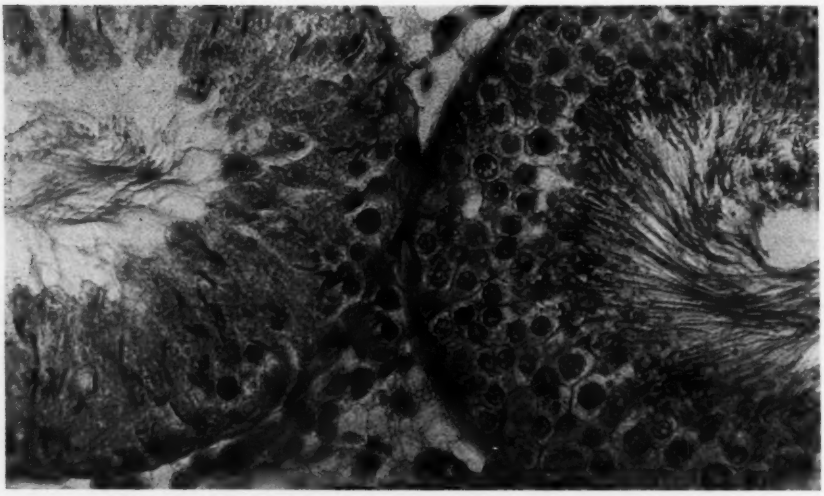

FIG. 10. Testis from rat 20 days after x-irradiation of the scrotal area. One tubule contains spermatids and sperm, while in the other virtually all spermatids have undergone maturation to sperm. ×500.

mature sperm, formed the entire thickness of the germinal epithelium (Fig. 9). Twenty days after irradiation many of the tubules revealed a picture similar to that seen on the 15th day, i.e., the presence of numerous normal appearing spermatids and sperm, although in some the spermatids were few and the sperm scattered (Fig. 10). An analogous microscopic

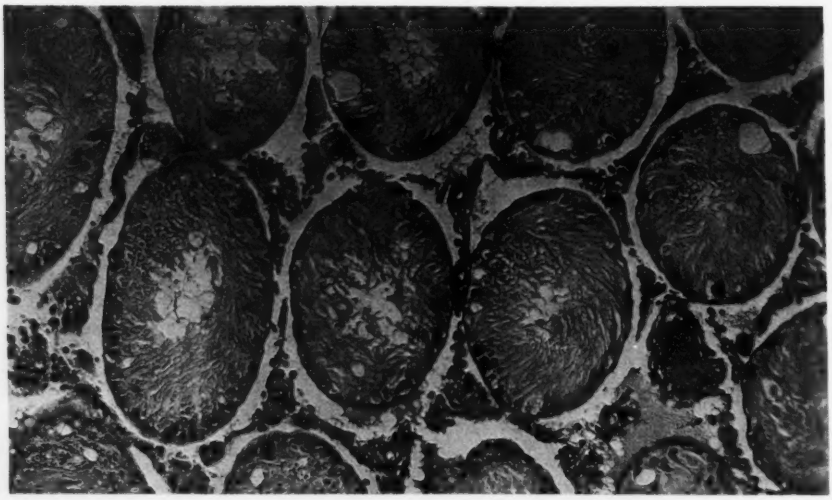

FIG. 11. Testis from rat 35 days after x-irradiation of scrotal area. Except for occasional residual sperm, all germ cells have disappeared.

picture was observed in testes of animals sacrificed 25 days after irradiation, except that many tubules showed a markedly decreased number or an absence of spermatids and sperm. By the 35th day after irradiation, the tubules contained only Sertoli cells with occasional spermatozoa and rare spermatids. As was true at all stages, the interstitial areas appeared to be undamaged by irradiation (Fig. 11).

DISCUSSION

In earlier studies we had demonstrated that the Leydig cells, the Sertoli cells, and the spermatogonia probably are not directly concerned with the production of hyaluronidase (3). These studies also suggested that cells beyond the stage of spermatocytes are closely associated with the presence of hyaluronidase in the testes. However, testicular changes induced by the experimental conditions examined were not selective enough, so far as elimination or preservation of spermatocytes is concerned, to permit us to draw more than tentative conclusions. The finding that hyaluronidase concentration was low in testes in which spermatocytes were either numerous or sparse could thus not be regarded as conclusive evidence either for or against the production of the enzyme by such cells. The necessary experiment was one in which not only the spermatocytes, but other germinal cells as well, would be controlled in predictable fashion.

It has been shown that the administration of furadroxyl, in rats, produces a uniform arrest of spermatogenesis at the stage of primary spermatocytes (4, 5). Furadroxyl treatment seemed to be particularly adapted to our problem, since control of the different phases of spermatogenesis could be maintained more accurately than in the procedures used in our earlier experiments.

In the present study a significant drop in hyaluronidase concentration was observed as early as the 10th day of treatment with furadroxyl. The microscopic studies of such testes revealed a decrease of young spermatids, but normal numbers of primary spermatocytes. Even more striking were the findings in animals sacrificed after 15 days of treatment. Spermatids and sperm were completely absent in most tubules, and in the remainder were markedly decreased and frequently showed profound cytopathologic changes. The concentration of hyaluronidase in these testes was negligible. On the 20th and 30th days of treatment, primary spermatocytes continued to be present in relatively large numbers and assays for hyaluronidase revealed complete lack of the enzyme.

Further confirmation of this pattern of correlation between hyaluronidase concentration and the cellular population of the tubules was obtained from studies of the enzyme concentration during the period of recovery after furadroxyl treatment. On the 20th day of recovery, when relatively normal numbers of dividing spermatocytes but only very few young (Golgi phase) spermatids were present, no hyaluronidase could be detected in the

testicular tissue. On the 25th day of recovery, with the appearance of young spermatids in many of the tubules and more mature spermatids (cap phase) in some of them, small quantities of the enzyme were present. It is obvious that in these testes the primary spermatocytes could not have been in a state of arrest, since they were able to produce, in many of the tubules, a generation of spermatids; nevertheless, no substantial rise in hyaluronidase concentration could be detected. On the other hand, the increase in number of spermatids, with progression of recovery, was associated with an essentially parallel increase in enzyme concentration. After 60 days of recovery the histological picture of the testes had returned to normal and the enzyme content also approached normal levels. These observations indicate that the spermatocytes are not directly related to the production of hyaluronidase, whereas a close relationship does appear to exist between the spermatid population and concentration of the enzyme.

Testicular recovery in animals treated concomitantly with furadroxyl and estrogen is much more rapid and uniform than recovery after treatment with furadroxyl only (6). This observation was utilized and confirmed in the present study. As early as the 15th day of recovery, some tubules contained spermatids in the Golgi phase of development and traces of hyaluronidase could be detected. On the 20th day of recovery, most of the tubules contained spermatids in the cap phase and the enzyme concentration had risen to about 20 per cent of normal. Subsequent increase in hyaluronidase levels paralleled further recovery of the germinal epithelium beyond the spermatocyte stage; and on the 40th day the content of enzyme had reached normal limits with the corresponding microscopic picture showing return to normal spermatogenesis.

If the spermatids are the sole producers of testicular hyaluronidase, any condition which eliminates the spermatogonia and spermatocytes without injuring the spermatids, should fail to cause a decrease in the enzyme concentration. Actually, there should be some *increase* since in such a condition the relative number of enzyme producing cells per unit of testicular weight would be greater than in normal testes.

In testing this hypothesis, advantage has been taken of the repeatedly demonstrated phenomenon that x-rays produce an irreversible damage to the spermatogonia without direct injury to the remaining elements of the spermatogenic epithelium. Our experiments showed that on the 10th day after irradiation, the hyaluronidase concentration of the testes increased well above the normal value, while the testicular weights decreased and spermatogonia were absent. Fifteen days after irradiation, the enzyme concentration remained above the normal value, testicular weights decreased further, and microscopic study showed the absence of spermatogonia and, in most tubules, of spermatocytes. These observations provide further evidence that spermatocytes and spermatogonia almost certainly are not the site of production of hyaluronidase. Twenty days after irradiation

many tubules contained no germinal elements other than varying numbers of sperm, although many others showed maturing spermatids, a histological picture associated with hyaluronidase levels significantly below normal levels. The concentration of hyaluronidase decreased progressively with the disappearance of spermatids, so that on the 35th day after irradiation, when the enzyme content was negligible, the tubules contained only Sertoli cells, occasional sperm and rare spermatids. These observations on irradiated testes appear to provide significant evidence for a direct relationship between the spermatid population and the concentration of hyaluronidase and suggest very strongly that the enzyme is produced during spermatid maturation.

The mature spermatozoon of various species has been shown to contain hyaluronidase (7, 8, 9). Nevertheless it would appear unlikely that this cell actually forms an appreciable amount of the enzyme, particularly in view of the fact that the hyaluronidase concentration in the testes may reach rather high concentrations before a significant number of sperm is formed, e.g., during recovery from furadroxyl treatment, or in the course of testicular maturation (1, 2). Therefore, since hyaluronidase is actually carried by spermatozoa, the enzyme produced by the spermatid must be passed along to the mature spermatozoon.

SUMMARY

Testicular structure and hyaluronidase concentration were correlated in rats (a) treated with furadroxyl, (b) recovering from furadroxyl treatment, (c) recovering from furadroxyl and estrogen treatment, and (d) x-irradiated with 600 r delivered to the scrotal area. In all of these conditions the concentration of the enzyme paralleled that of the spermatids in the testicular tubules.

On the basis of the evidence presented in this paper and in earlier related studies, it is suggested that the spermatid is the testicular cell responsible for the production of hyaluronidase.

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STUDIES ON THE METABOLISM OF THYROTROPIN[1]

HAROLD A. LEVEY[2] AND DAVID H. SOLOMON

Departments of Physiological Chemistry and Medicine, School of Medicine, University of California Medical Center, Los Angeles 24, California.

RAPID disappearance from the plasma of large exogenous doses of heterologous thyrotropin has been reported in man (1), rabbit (2), and rat (3, 4). Less than 5% of the administered dose of thyrotropin remained in rat plasma one hour after intravenous injection (3). Both thyroid (5–7) and liver (8) have been implicated, under *in vitro* conditions, as the site of inactivation of thyrotropin, although there is *in vivo* evidence that inactivation may be related primarily to general body metabolism rather than to the function of the thyroid gland *per se* (4). It has been reported that thyrotropin accumulates in liver (9) or kidney (10) within a few minutes after intravenous injection, and persists in the thyroid gland as long as 24 hours after injection (9). However, it must be pointed out that in one of these studies (9), S^{35}-labeled thyrotropin was detected in the organs by its radioactivity rather than by biological activity.

In view of this sparse and controversial literature, investigations were undertaken to study the time course of disappearance of exogenous thyrotropin from rat plasma under different experimental conditions, and to obtain information on the distribution of this hormone in the body.

MATERIALS AND METHODS

In vivo studies

The rate of disappearance and distribution of intravenously administered thyrotropin were studied in male Sprague-Dawley rats weighing 150–250 grams. Comparisons were made among intact, thyroidectomized, thyroidectomized-thyroxine treated, eviscerated, and eviscerated-hepatectomized animals. Thyroidectomized rats were used 3 weeks postoperatively; some of these animals were maintained on 25 μg. of dl-thyroxine daily for the last 2 weeks. Evisceration or evisceration and hepatectomy[3] were carried out on animals fasted overnight (11); the kidneys were not removed.

Received July 27, 1956.

[1] This investigation was supported by research grants RG-3869 from the Division of Research Grants and A-271 from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

[2] Present address: Department of Physiology, State University of New York, State University Medical Center at New York City, Brooklyn 3, New York.

[3] In the animals with intact livers, the hepatic artery provided the sole blood supply. The splenic and left gastric branches of the coeliac axis, and the gastro-duodenal branch of the hepatic artery were tied and cut. When the liver was removed, the entire coeliac axis was ligated and cut. It is understood that liver function was probably impaired in the animals which had to rely on the limited blood supply from the hepatic artery. BMR was determined on each animal immediately before the operation and as soon as

Experimental procedure

A freshly prepared saline solution of 2 to 6 U.S.P. units of thyrotropin[4] was administered via the saphenous vein and the animals were exsanguinated from the abdominal aorta under ether anesthesia at intervals varying from 2 to 120 minutes later. Blood was collected in citrated syringes and plasma prepared. The carcasses were perfused through the heart with several 10 cc. aliquots of saline in order to remove residual blood. The liver or kidneys were removed for separate assay in some experiments. The carcasses were then skinned and the pituitary glands were removed and discarded. Urine was collected for 60 and 120 minutes from 2 animals kept in individual metabolism cages. At sacrifice, the bladder urine was removed with a syringe and added to the original collection.

Preparation of samples for assay

Plasma. Plasma was diluted with saline in order to bring the thyrotropin concentration into the effective dose-response range of the bioassay. The concentration of thyrotropin in U.S.P. units per cc. of undiluted plasma was multiplied by a factor of 2.4% of the body weight (12) to obtain the approximate total plasma content of thyrotropin.

Carcass. The perfused, skinned carcass was homogenized in a Waring Blendor with a cold, aqueous solution containing 1.0 gm. of sodium chloride and 0.25 gm. of glacial acetic acid per 100 cc. In experiments in which a single extraction was carried out, 60% of the initial carcass weight was assumed to be water and an amount of medium was added to make a total aqueous volume of 300 cc. The carcass homogenate was centrifuged in the cold at 3900 r.p.m. The supernate was then filtered through glass wool to remove suspended fat and debris and diluted with saline as required for assay. In some cases (see Table 3 below) the carcass was successively extracted with 3 aliquots of medium totalling 500 cc. The combined supernates were reduced to a volume of 40 cc. by rotary evaporation under reduced pressure.

Kidney and liver. These tissues were homogenized and extracted with 3 successive aliquots of 3 cc. of cold acidified saline. The combined supernates were made up to 10 cc.

Urine. Urine was filtered, diluted with saline, and injected into the assay animals without further treatment.

In vitro studies

Incubation. Incubation of 0.2 U.S.P. units of thyrotropin (Armour Thytropar) with 50 mg. of rat liver slices or thyroid tissue in 0.5 cc. of rat serum was carried out for 5 hours in Warburg flasks of 6 cc. capacity. Duplicate flasks were run for each tissue. Duplicate control flasks contained 0.2 U.S.P. units of thyrotropin and 0.5 cc. of serum but no tissue. The flasks were gassed with oxygen and maintained at 37° C. Oxygen

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it emerged from anesthesia following the operation. Five U.S.P. units of thyrotropin (Armour Thytropar) were injected after determination of post-operative BMR, and the animals were sacrificed one hour later. Survival was enhanced by the fasting period, which served to deplete the animals of their carbohydrate stores and enable them to adjust to fat utilization.

[4] Armour Thytropar, U.S.P. Thyrotropin Reference Substance, and rat anterior pituitary extract were used as indicated. The latter was prepared from several hundred pituitary glands collected from Sprague-Dawley and Long-Evans rats of both sexes. The pooled glands (free of the posterior lobes) were extracted with acidified saline and the extract kept frozen until use. The potency of the extract was maintained at about 2 U.S.P. units of thyrotropin per cc. by periodic addition of fresh material.

consumption was determined by the direct method of Warburg. At the end of the incubation period the serum media were assayed for thyrotropin.

Perfusion. The perfusion apparatus was designed by Dr. Josiah Brown after that of Hechter (13). A rabbit liver weighing 47 gm. was perfused via the hepatic portal vein at a rate of 80–100 cc. per minute. The blood was collected from the vena cava and recycled. The reservoir initially contained 325 cc. of citrated rabbit blood (255 cc. of blood and 70 cc. of Baxter A.C.D. Solution) to which were added 6.8 U.S.P. units of thyrotropin (Armour Thytropar). This initial concentration of thyrotropin was determined by bioassay of the pre-perfusion plasma. Samples of 6 cc. were taken from the reservoir at the start of the perfusion and at hourly intervals thereafter for 5 hours. Plasma was prepared and assayed for thyrotropin. The glucose content of the plasma was determined by the method of Durham *et al.* (14). Plasma volume was determined by hematocrit. All of the thyrotropin was assumed to be in the plasma. At the end of the run the liver was perfused with saline to remove residual blood and the gall bladder was removed. A saline extract of the liver was prepared as described above. The bile was diluted to 5 cc. The bile and liver preparations were assayed for thyrotropin.

Bioassay procedures

Chick P^{32} uptake method. This method was adapted from that of Greenspan *et al.* (15) with changes made in the time relationships of the injections. The test sample was administered by intracardiac injection (0.1 or 0.2 cc.) to day-old White Leghorn cockerels (5 or 6 per group). Ten microcuries of P^{32} were given subcutaneously in 0.1 cc. of saline 3 hours later. The chicks were killed by decapitation 3 hours after the P^{32} injection. The thyroid glands were removed, trimmed, dried on metal planchets, and their radioactivity determined with an end-window Geiger tube. A 3- or 4-point standard curve was run with each assay, covering a range of 0.3 to 4.0 U.S.P. milliunits of thyrotropin. Each unknown sample was injected at 2 to 4 levels of dilution.

Rat I^{131} uptake method. This method has previously been described in detail (16). Immature Sprague-Dawley rats (60–70 gm.) were maintained for 10 days on a diet of 0.05% thyroid powder in ground Purina Laboratory Chow. The test sample was administered via the saphenous vein in a volume of 0.5 cc. Twenty hours later, 2–4 microcuries of I^{131} were given intraperitoneally. The rats (5–6 per group) were sacrificed 4 hours after the I^{131} injection. The thyroid glands were removed, trimmed, dried on metal planchets, and their radioactivity determined with an end-window Geiger tube. A 4-point standard curve was run with each assay, covering a range of 0.5 to 25.0 U.S.P. milliunits of thyrotropin.

Where indicated below, unknown samples were assayed in parallel by both methods. Otherwise, the chick method was employed. Trial runs were carried out on many of the unknowns in order to ascertain the proper dilution of samples for the final assay.

The mean and standard error of the index of precision (λ) for 7 chick assays was 0.204 ± 0.029. When one of the best assays in this group was applied to the analysis of an unknown by a 2×2 design, the value of λ was 0.267. This meant that a total of 120 animals would be required for each 2×2 assay in order to set fiducial limits of error which would not exceed $\pm 20\%$ at the 95% level of probability. It was further noted that the log dose-response curve of the unknowns did not always parallel the standard curve, thus defeating the 2×2 design. For these reasons we chose to use smaller numbers of animals per group, and to calculate recovery by reading directly off the simultaneously determined standard curve. Entire experiments were repeated when necessary in order to reduce the overall error.

RESULTS

I. *Disappearance of homologous and heterologous thyrotropin from rat plasma*

Table 1 describes the recovery of thyrotropic hormone activity from rat plasma at varying time intervals after intravenous administration of 2.0 U.S.P. units of Armour Thytropar or 1.2 equivalent U.S.P. units of rat anterior pituitary extract. Thyrotropin recovery was generally higher when determined by the rat assay. There was a suggestively slower disappear-

TABLE 1. DISAPPEARANCE OF HOMOLOGOUS AND HETEROLOGOUS THYROTROPIN FROM RAT PLASMA

Thyrotropin preparation administered[1]	Minutes from thyrotropin injection to sacrifice	Number of rats per group[2]	% of recovery of thyrotropin	
			By chick assay	By rat assay
2.0 U.S.P. units of Armour Thytropar	2	2	81.5	113
	15	3	18.3	33
	60	3	1.1	7
1.2 equivalent U.S.P. units of rat anterior pituitary extract	2	3	61.5	52
	15	3	30.6	43
	60	3	2.1	10

[1] Administered via saphenous vein in 0.5 cc. of saline.
[2] Two cc. of plasma from each rat pooled per group.

ance of rat pituitary thyrotropic activity as compared with that of Thytropar at 15 and 60 minutes. The differences were demonstrated with both assay methods. The more rapid disappearance of the rat activity at 2 minutes cannot be interpreted. Erratic 2 minute recoveries were observed in these experiments, probably because at the early time periods the disappearance rate follows an extremely steep curve. Employing the chick assay method, a total of 10 separate determinations of thyrotropin recovery at 60 minutes in the normal rat was carried out with Thytropar (Tables 1, 2, and 3) and 3 with rat pituitary extract (Tables 1 and 4). The mean per cent recoveries and standard errors of the mean were 1.5 ± 0.3

TABLE 2. DISTRIBUTION OF THYROTROPIN IN THE NORMAL RAT

Minutes from thyrotropin[1] injection to sacrifice	% recovery of thyrotropin by chick assay			
	Plasma	Carcass[3]	Liver	Urine
2[2]	65.2	44.2	4.1	—
15	19.5	59.0	3.5	—
60	0.7	23.1	0	1.4
120	0	16.5	0	5.3

[1] 6.0 U.S.P. units of Armour Thytropar administered via the saphenous vein.
[2] A single animal was observed at each time interval.
[3] Prepared by single extraction method (see Materials and Methods).

TABLE 3. SUMMARY OF PLASMA, KIDNEY, AND CARCASS RECOVERIES OF INTRAVENOUSLY ADMINISTERED THYROTROPIN

Minutes from thyrotropin[1] injection to sacrifice	% recovery of thyrotropin by chick assay			
	Plasma	Kidney	Carcass	Kidney plus carcass[2]
2	65.2	—	—	44.2
5	32.5	5.7	20.7	26.4
5	39.8	3.6	17.6	21.2
5	31.2	3.9*	—	—
5	31.7	4.6*	—	—
5	31.2	5.0*	—	—
5	39.8	10.6*	—	—
15	19.5	—	—	59.0
60	0.7	—	—	23.1
60	1.3	—	—	19.2
60	1.1	—	—	20.9
60	1.4	—	—	36.6
60	0.8	—	—	21.0
60	1.2	4.5	9.9	14.4†
60	0.4	3.3	9.2	12.5†
60	3.0	2.7	14.2	16.7†
120	0	—	—	16.5

[1] Each rat received 5–6 U.S.P. units of either Armour Thytropar or Thyrotropin Reference Substance.

[2] Where kidney was homogenized together with carcass, the observed recovery is recorded in this column. Where kidney was assayed separately from carcass, the sum of the two recoveries is recorded.

* Non-perfused.

† Carcass extracts were prepared by the triple extraction method (see Materials and Methods). All others were prepared by the single extraction procedure.

and 6.1 ±2.4, respectively. These values were significantly different ($P < 0.01$).

II. *Tissue distribution of thyrotropin*

Table 2 shows recovery of thyrotropic activity from plasma, carcass, liver, and urine at varying time intervals after intravenous administration of 6.0 U.S.P. units of Thytropar. Activity in plasma declined progressively until it was no longer detectable 120 minutes after administration. A sizeable portion of the original activity remained in the perfused carcass[5] long after it had disappeared from the plasma. A lesser amount of activity was recovered from liver at the early time intervals but was gone in 60 minutes. Urinary excretion of thyrotropic activity amounted to 5.3% in 120 minutes.

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[5] We wish to correct a previous preliminary report on this work (23), which stated that thyrotropin was not detectable in carcass extracts following intravenous injection of the hormone. The statement was based on early observations made with a technique of carcass extraction which later was shown to result in very low recovery of added thyrotropin.

An average of 5.5% and 3.5% of the administered dose was recovered from kidney 5 and 60 minutes, respectively, after intravenous injection of 5–6 U.S.P. units of thyrotropin (Table 3). Kidney recoveries at 5 minutes were similar in perfused and non-perfused animals, indicating that thyrotropin was fixed in the kidney as it was in the carcass. A summary of all the assays performed on carcass extracts from normal rats is also presented in Table 3. The persistence of activity in the carcass, as contrasted with plasma, is well demonstrated by these data. It is to be noted that the 3 carcass extracts prepared by the triple extraction method yielded consistently lower recoveries of thyrotropin than those prepared by the single extraction method.

No thyrotropic activity was present in extracts of the carcass of a normal, untreated rat or in washed, laked erythrocytes shortly after administration of thyrotropin. As a check on the method of preparation of the tissue extracts, a rat was skinned, homogenized, and extracted 2 minutes after administration of thyrotropin. Only the pituitary gland of this animal was removed. All of the administered dose of thyrotropin was recovered in the extract, indicating that no inactivation occurred during the period of homogenizing and centrifuging. This process was repeated with another rat 60 minutes after thyrotropin administration and only 32.2% of the administered dose was recovered.

III. *Effect of thyroidectomy on recovery of thyrotropin from plasma and carcass*

As determined by the chick assay method, recovery of activity from plasma 15, 30, and 60 minutes after injection of 1.5 equivalent U.S.P.

TABLE 4. EFFECT OF THYROIDECTOMY ON RECOVERY OF INTRAVENOUSLY ADMINISTERED THYROTROPIN FROM RAT PLASMA

Treatment of rats	Minutes from thyrotropin[1] injection to sacrifice	Number of rats per group[2]	% recovery of thyrotropin	
			By chick assay	By rat assay
Unoperated controls	15	2	27.9	34.9
	30	2	21.1	16.5
	60	2	10.6	4.3
	60	2	5.7	6.4
Thyroidectomized 20 days	15	2	26.4	28.7
	30	2	17.9	27.1
	60	2	9.2	14.1
	60	2	10.2	10.8
Thyroidectomized 20 days plus 20 μg. DL-thyroxine daily for last 18 days	15	2	20.8	29.2
	30	2	21.0	18.4
	60	2	6.2	7.9
	60	2	6.4	6.0

[1] Each rat received 1.5 equivalent U.S.P. units of rat anterior pituitary extract in 0.5 cc. of saline via the saphenous vein.
[2] Two cc. of plasma from each rat pooled per group.

TABLE 5. EFFECT OF THYROIDECTOMY ON 60 MINUTE RECOVERY OF THYROPROTEIN FROM RAT PLASMA AND CARCASS

Treatment of rats	% recovery of thyroprotein[1] by chick assay	
	Plasma	Carcass[2]
Unoperated controls	1.4	36.6
	1.1	20.9
	0.8	21.0
Thyroidectomized 20 days	2.2	25.2
	1.6	28.0
	1.0	23.1
Thyroidectomized 20 days plus 20 μg. dl-thyroxine daily for last 9 days	0.8	27.0
	0.3	26.8

[1] 5 U.S.P. units of Armour Thytropar injected via the saphenous vein.
[2] Prepared by single extraction method (see Materials and Methods).

units of rat pituitary extract was relatively unaffected by thyroidectomy or thyroidectomy plus thyroxine administration (Table 4). When the same samples were assayed by the rat method, a slower disappearance of thyrotropin was observed in the thyroidectomized groups 30 and 60 minutes after injection. Recovery of activity from carcass extracts 60 minutes after injection of 5.0 U.S.P. units of Thytropar was unchanged by thyroidectomy (Table 5). In this experiment, slowest disappearance from plasma was seen in the thyroidectomized group, and fastest disappearance was seen in the thyroidectomized group treated with thyroxine, but the differences were small. The BMR of the untreated thyroidectomized rats fell to an average of 36% below normal.

IV. *Effect of hepatectomy and evisceration on disappearance of thyrotropin from plasma*

Since the liver has been implicated as a possible site of inactivation of thyrotropin, at least *in vitro*, disappearance studies were conducted on animals which had been either eviscerated (with intact liver) or eviscerated and hepatectomized. The results were generally unsatisfactory because of the varying degrees of blood loss and shock accompanying the operations. In addition, the post-operative BMR of these animals declined 40–50% below the pre-operative level, introducing a variable which might of itself have accounted for the observed alteration in the rate of thyrotropin disappearance from plasma. The recoveries, based on a plasma volume equal to 2.4% of the post-operative body weight of these rats, were in the range of 20–40% of the administered dose of thyrotropin 60 minutes after injection, as compared with less than 5% in the normal rat. The recovery in eviscerated rats with intact liver was equal to that in eviscerated and hepatectomized animals. The role of the intestine as a possible site of inactivation of thyrotropin has not been studied.

V. *In vitro studies on thyrotropin inactivation by liver and thyroid tissue*

In the incubation experiment (see Materials and Methods), recovery of thyrotropin in U.S.P. units amounted to 0.218 and 0.233 in the control flasks, 0.232 and 0.240 in the thyroid incubates, and 0.213 and 0.230 in the liver incubates. The control flasks also served as thermobars for determination of oxygen consumption. The rates of oxygen consumption for the fifth hour were 86% and 81% of the first hour values for the duplicate thyroid incubations, and 70% and 65% of the first hour values for the liver incubations.

In the perfusion study (Table 6), 29% of the total thyrotropin was inactivated by the end of the second hour with no further inactivation out to

TABLE 6. INACTIVATION OF THYROTROPIN BY LIVER PERFUSION[1]

Sample	Total content of thyrotropin (U.S.P. units)	Total glucose in plasma (mg.)
Control rabbit plasma before addition of thyrotropin	subthreshold	—
0 hour plasma	6.80	1112
1 hour plasma	6.22	1334
2 hour plasma	4.86	1127
3 hour plasma	4.81	1093
4 hour plasma	4.80	945
5 hour plasma	4.81	486
Liver at end of perfusion	0.157	
Bile at end of perfusion	0.015	

[1] See Materials and Methods.

5 hours. After an initial release of carbohydrate from liver to blood, glucose continued to disappear from the plasma for as long as 5 hours, indicating that the liver was probably still in good condition. Recovery of thyrotropin from liver and bile amounted to 2.3% and 0.2% of the original amount, respectively, and ruled out the possibility that thyrotropin was concentrated from the plasma into liver or bile. It must be pointed out that the experiment was not controlled by a repeat perfusion in the absence of the liver.

DISCUSSION

The rapid disappearance of exogenous thyrotropin from the plasma might be due to 1) destruction of heterologous thyrotropin as a result of a foreign protein reaction, 2) destruction or inactivation of homologous thyrotropin by one or more organs or tissues, 3) urinary excretion of thyrotropin, 4) hepatic or fecal excretion of thyrotropin, or 5) distribution of thyrotropin from the plasma into extra- or intracellular compartments, not necessarily accompanied by inactivation.

In the present study, a significant difference was seen in the rate of dis-

appearance of heterologous and homologous thyrotropin from rat plasma, but the difference was not large enough from a physiological standpoint to support the possibility that a foreign protein reaction is the primary cause of the rapid removal of biological activity from blood. It has been previously reported (17) that there was no difference in I^{131} release curves in the rabbit following intravenous administration of either beef thyrotropin or rabbit pituitary extract.

Inactivation of thyrotropin *in vitro* has been reported in the presence of minced guinea pig liver (8), macerated beef thyroid (5), rabbit thyroid slices (18), cultures of rabbit thyroid and lymphatic tissue (6), and human thyroid tissue (7). *In vivo* studies, however, have essentially ruled out the thyroid as an important factor in thyrotropin inactivation. D'Angelo (3, 4) demonstrated that the hypophysectomized rat did not inactivate exogenous thyrotropin as rapidly as the normal rat, but that treatment of the former with thyroxine or triiodothyronine restored the rate of inactivation to normal. This suggested that it was the metabolic rate rather than the presence or absence of the thyroid which was the determining factor in thyrotropin inactivation. More recently, it was found that rabbits thyroidectomized 3 days before thyrotropin administration disposed of the hormone at a normal rate, while those thyroidectomized 3–5 months previously showed a delayed disappearance of exogenous thyrotropin from plasma (2). These findings closely support D'Angelo's conclusions. In the present studies, the rate of disappearance of thyrotropin from plasma was similar in normal, thyroidectomized, and thyroidectomized-thyroxine treated rats (although it tended to be slightly slower in the thyroidectomized groups), even though the BMR of the thyroidectomized animals was depressed about 36%. These observations confirm the view that the thyroid is not primarily responsible for thyrotropin inactivation, but do not support the idea that the rate of inactivation is related to total body metabolism.

We were unable to obtain clearcut evidence for hepatic inactivation of thyrotropin. Reduced thyrotropin inactivation was observed following evisceration and hepatectomy, but these findings were unreliable because of uncontrolled experimental conditions. The intestinal tract remains as a possible site of inactivation. No thyrotropin inactivation was seen after a 5 hour incubation with liver slices, even though oxygen consumption was well maintained for the entire period. In a single perfusion experiment, 29% of the circulating thyrotropin was inactivated by a rabbit liver in 2 hours. This amounts to approximately 0.3% per gm. per hour and would be negligible if considered in the light of the overall *in vivo* rate of inactivation.

In the present studies, 5.3% of the administered dose of thyrotropin was excreted in the urine 2 hours after injection. Urinary excretion of thyro-

tropin has also been reported in patients with myxedema (19, 20), and in totally thyroidectomized patients (21). Ordinarily, thyrotropin activity is not detectable in normal urine, but this might be the combined result of inadequate means of concentrating the material for injection, and insufficient sensitivity of the assay method. Promising results have been reported with ultrafiltration of normal human urine through a collodion membrane (22). Material so concentrated contained thyrotropic activity amounting to 0.01–0.025 U.S.P. units per 24-hour specimen. Hepatic and fecal excretion of thyrotropin have not been investigated.

One hour after intravenous administration of thyrotropin, over 20% of the injected dose was regularly recovered in extracts of the rat carcass, even following saline perfusion to remove residual blood. This finding points to the possibility that thyrotropin might be sequestered in tissues and released gradually back into the blood stream, maintaining a low level of activity which, in the normal animal, does not exceed the threshold sensitivity of the majority of the methods for the bioassay of thyrotropin. Less than 5% of the injected dose of thyrotropin was recovered from liver extracts 15 minutes after administration of the hormone, and none was detectable in the liver in an hour. Kidney also contained less than 5% of the administered thyrotropin 5 minutes after injection, but activity persisted in this organ for at least an hour. In the group studied 5 minutes after thyrotropin injection, if the water content of the kidney were considered as the approximate space of distribution for thyrotropin, then the ratio between the activity per cc. of kidney water and activity per cc. of plasma did not exceed unity. This is in contrast to a recent report by Kassenaar *et al.* (10), who obtained 5 minute kidney recoveries of 10–30% of the administered dose of thyrotropin, and kidney/blood activity ratios of 4 to 7. The only major difference in experimental conditions was in the use of Organon thyrotropin in the latter study. It is conceivable that proteinaceous materials with the same biological activity but prepared by different methods might have different affinities for renal tissue.

The combined recoveries of beef thyrotropin one hour after injection averaged 20.7% in carcass, none in liver, 3.5% in kidney, 1.2% in blood, and 1.4% in urine. These results compare favorably with a value of 32.2% of the administered dose recovered from the whole rat (minus skin and pituitary gland) one hour after injection of thyrotropin.

It may be concluded from the present studies that the site of inactivation of exogenous thyrotropin is still unknown. Thyroid and liver probably play no specific role in this process. Urinary excretion appears to be of minor significance. Inactivation might occur slowly in all of the tissues of the body. The possibility remains that the intact kidney may be highly active in the destruction of thyrotropin, although kidney slices (18) and cultures of kidney tissue (6) do not appear to have this capacity. The an-

swer to this question must await studies on perfusion of the isolated kidney with thyrotropin, and a comparison of thyrotropin disappearance in normal and nephrectomized animals.

SUMMARY

Distribution and inactivation of intravenously administered thyrotropin have been studied in the rat. An hour after injection over 98% of the thyrotropic activity had disappeared from plasma. Activity disappeared at a significantly slower rate following injection of rat pituitary extract than after administration of beef thyrotropin, but the difference was not large enough to explain the rapid loss of activity on the basis of a foreign protein reaction.

The rate of disappearance was not significantly affected by thyroidectomy, demonstrating that the thyroid gland is not a major site of thyrotropin inactivation.

Injected thyrotropin was distributed from plasma to tissues so that in one hour over 20% of the administered dose was recovered from the carcass, 3.5% from the kidneys, and 1.4% had been excreted in the urine.

No inactivation of thyrotropin was observed *in vitro* after incubation with rat liver and thyroid tissue, but 29% disappeared in 2 hours of perfusion through an isolated rabbit liver.

Recovery of injected thyrotropin was greater in animals which had been eviscerated and hepatectomized, but the poor conditions of these animals precluded definitive conclusions regarding the role of the removed organs in thyrotropin inactivation.

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Acknowledgment

The authors gratefully acknowledge the technical assistance of Miss Ruth Greene, Miss Alice Kittinger, Mrs. Leona Monroe, and Mrs. Ruth Upton; and the valuable aid of Dr. Josiah Brown of the Department of Medicine in the perfusion study.

THE EFFECT OF SOME NATURAL AND SYNTHETIC SEX HORMONES ON ADRENAL CORTICAL SECRETION *IN VITRO*

KENNETH W. McKERNS[1]

Department of Physiology, University of St. Andrews, Fife, Scotland

WE HAVE previously studied the long-term effects of depot estradiol and testosterone on pituitary-adrenal function in the intact rat (1). It is of considerable importance to know if in addition to mediation through the pituitary, there is any direct action of sex hormones on adrenal cortical function. Several estrogens and androgens, natural and synthetic, have been examined in an *in vitro* system.

MATERIALS AND METHODS

Both Wistar and Sprague-Dawley rats of 100–150 gm. have been used. The animals were littermate related in the 16 and 32-rat experiments—8 litters of 2 or 4 littermates being used. When only 8 rats are used in an experiment they need not be littermates since the adrenal tissue is distributed, a quarter from each adrenal pair to each of eight tubes.

The rats are anesthetized with Nembutal, and the adrenals removed and trimmed free of adhering tissue. Each adrenal of a pair is quartered and a piece placed in each of eight sectors of a buffer moistened filter paper in a covered petri dish. This distribution is repeated for each of the eight rats so that each sector contains a quarter of either a left or right adrenal from each rat. This system is repeated either once more for the 16-rat or three times for the 32-rat experiments. Littermates are used in these cases.

The tissue in a sector is weighed (approximately 25 mg.) and transferred to a small flat bottom tube containing 1 ml. of Krebs-Ringer bicarbonate buffer made up with 200 mg. % glucose. Each tube is shaken for one hour on a rack in a Warburg apparatus at 37° C in equilibrium with 95% oxygen, 5% carbon dioxide, as previously described. (2) The buffer is aspirated from the gland pieces and 1 ml. fresh buffer or 1 ml. buffer containing the steroid under study is added for an additional two hours. If a steroid was not readily water soluble it was dissolved in 0.5–1.0 ml. propylene glycol and dispersed into 50 ml. buffer so that each ml. contained the required amount of steroid. Corticotrophin, if used, was added in 0.02 ml. buffer. The corticotrophin used was a purified preparation of 40 I.U./mg. prepared by the method of McKerns (3) and assayed against both the first U.S.P. Provisional Reference Standard and the new U.S.P. Standard, by the method of McKerns and Nordstrand (4).

The buffer from the second incubation is extracted with 2 ml. methylene chloride by shaking for 1 min. The aqueous layer is aspirated off and 5–10 mg. anhydrous sodium sulfate is added. The methylene chloride can then be transferred to 1.5 ml. silica cells. The absorption at 240 mμ due to the α, β unsaturated ketonic group of the corticoids is

Received August 3, 1956.

[1] Present address: Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y.

estimated from the corrected optical density formula O.D. corrected = $O.D._{240} - O.D._{260}$ (5). A standard curve was prepared for optical density in terms of μg. cortisone acetate. The appropriate buffer containing the test substances was extracted and the methylene chloride used as blank. The use of methylene chloride for extraction and measurement of corticoids reduces the interference effect at 240 mμ to a small absorption parallel to the horizontal axis (6). A comparison between ultra violet absorption estimations of α, β unsaturated ketonic and tetrazolium reducing steroids has been made by Elliott, Birmingham, Schally and Schönbaum (7). Chromatographic separations were made of extracts from adrenal incubates and measured by both methods (8).

RESULTS AND DISCUSSION

In Table 1 it is shown that estradiol even at a high dose level (10 μg./25 mg. adrenal tissue) had no effect on the corticotrophin stimulated adrenal corticoid secretion.

In Table 2 it can be seen that the addition of testosterone (1 μg./25 mg. adrenals) also did not affect either the unstimulated or the corticotrophin stimulated secretion of adrenal cortical tissue.

Again in Table 3 a and b high levels of added estradiol or testosterone did not interfere with adrenal steroid metabolism. However a substituted estrogen, ethinyl estradiol at 50 μg./45 mg. adrenals (male) and 50 μg./78 mg. adrenals (female) caused a 20% reduction in corticotrophin stimulated

TABLE 1. EFFECT OF ESTRADIOL 17 SODIUM SULFATE (10 μG./25 MG. ADRENALS) ON ADRENAL CORTICOID SECRETION IN FEMALE SPRAGUE-DAWLEY RATS *in vitro*. RESULTS ARE GIVEN AS μG. CORTICOIDS/100 MG. ADRENALS

	1 Corticotrophin 0.005 I.U./100 mg.	2 Corticotrophin 0.005 I.U./100 mg. plus estradiol	3 Corticotrophin 0.015 I.U./100 mg.	4 Corticotrophin 0.015 I.U./100 mg. plus estradiol	Totals
	21.4	22.3	30.2	30.6	104.5
	23.1	23.4	31.6	30.1	108.2
	22.0	21.8	30.2	33.8	107.8
	23.4	19.6	25.4	25.9	94.3
Totals	89.9	87.1	117.4	120.4	
Means	22.5	22.0	29.4	30.1	

Analysis of variance

Nature variation	Degrees freedom	Sum of squares	Mean square
Total	15	268.00	
Treatments	3	186.00	
Litters (down)	3	43.00	
Error S^2	9	39.00	4.3

$$\text{S.E. mean} = \pm\sqrt{\frac{s^2}{n}} = 1.03$$

$$t = \frac{\text{difference between means}}{\sqrt{2}.\text{S.E.}}$$

t between 1 & 2 = 0.49 not significant $P > 0.6$
t between 3 & 4 = 0.68 not significant $P > 0.5$

TABLE 2. EFFECT OF TESTOSTERONE SODIUM SULFATE (1 μG./25 MG. ADRENALS ON ADRENAL CORTICOID SECRETION IN FEMALE SPRAGUE-DAWLEY RATS *in vitro*. RESULTS ARE GIVEN AS μG. CORTICOIDS/100 MG. ADRENALS

	1 Unstimulated	2 Unstimulated plus testosterone	3 Corticotrophin 0.015 I.U./100 mg.	4 Corticotrophin 0.015 I.U./100 mg. plus testosterone	Totals
	26.1	25.2	38.5	34.4	124.2
	24.0	28.0	39.0	39.6	130.6
	22.5	22.6	27.7	34.4	107.2
	21.8	22.2	31.8	33.7	109.5
Totals	94.4	98.0	137.0	142.1	
Means	23.6	24.5	34.3	35.5	

Analysis of variance

Nature variation	Degrees freedom	Sum of squares	Mean square
Total	15	704.00	
Treatments	3	443.00	
Litters (down)	3	129.00	
Errors S^2	9	132.00	15.0

S.E. mean = 1.93

t between 1 & 2 = 0.47 not significant P > 0.6

t between 3 & 4 = 0.62 not significant P > 0.5

TABLE 3. EFFECT OF STEROID HORMONES ON CORTICOTROPHIN STIMULATED CORTICOID SECRETION OF WISTAR RAT ADRENALS *in vitro*

Experiment	Rat sex and number	Amount of compound added to experimental	Average weight adrenals	Corticotrophin added I.U./100 mg. adrenals	μg. corticoid/100 mg. adrenals: Control	μg. corticoid/100 mg. adrenals: Experimental	S.E. mean	t	Significant difference
a	8 M	estradiol 17 sodium sulfate (20 μg.)	60 mg.	0.040	17.7	18.5	0.73	0.90	none P > 0.8
					18.8	20.5			
					15.6	16.8			
					18.5	11.2			
				means	17.7	16.8			
b	8 M	testosterone sodium sulfate 20 μg.	52 mg.	0.045	13.5	14.0	0.70	0.30	none P > 0.7
					10.8	13.5			
					17.1	16.5			
					20.8	18.5			
					15.6	15.5			
c	4 M	ethinyl estradiol 50 μg.	45 mg.	0.045	M 23.5	17.5			20% reduction P < 0.01
	4 F		78 mg.		M 19.3	16.3			
					F 19.8	15.9			
					F 19.7	16.3	0.47	6.2	
				means	20.6	16.5			
d	8 F	stilbestrol 25 μg.	60 mg.	0.045	11.1	6.4			37% reduction P < 0.001
					12.0	7.4			
					11.5	7.3	0.013	24.0	
					11.9	8.0			
				means	11.6	7.3			
e	8 F	stilbestrol 25 μg.	60 mg.	0.045	9.9	2.6	0.27	17.54	67% reduction P < 0.001
					10.3	4.6			
					10.5	4.2			
					12.3	5.1			
				means	10.8	4.1			

total corticoid output in both the male and female adrenals. A synthetic estrogen of different structure from the natural hormones, stilbestrol, at 25 μg./60 mg. adrenals caused an even greater reduction (37%) in the corticotrophin stimulated corticoid output (d). The effect of stilbestrol is one of reversible inhibition. Experiment e is the corticoid output from the same glands as d. Fresh buffer was added to the glands and incubation was continued for 15 minutes. The buffer was discarded and buffer containing stilbestrol added to the former control series while the former experimental became the control. In Experiments d and e incuba-

TABLE 4. TRANS DEHYDRO ANDROSTERONE GLUCOSIDE AND ETHINYL ESTRADIOL EFFECT ON *female* SPRAGUE DAWLEY RAT ADRENAL CORTICOID SECRETION *in vitro*. RESULTS AS μG. CORTICOIDS/100 MG. ADRENALS

	1 *U*	2 *U+A*	3 *U+E*	4 *L*	5 *L+A*	6 *H*	7 *H+A*	8 *H+E*
	22.1	24.6	19.1	23.6	31.6	29.2	33.1	20.8
	21.4	29.4	15.6	28.2	29.7	22.7	32.8	23.7
	20.2	24.2	15.7	23.9	27.2	25.2	27.9	19.8
	20.7	29.2	16.6	28.2	36.6	29.2	34.2	23.0
Means	21.1	26.9	16.8	26.0	31.3	26.6	32.0	21.8

S.E. mean = 1.07

t between 1 & 2 = 3.83 $P < 0.001$
t between 1 & 3 = 2.93 $P < 0.01$
t between 4 & 5 = 3.50 $P < 0.01$
t between 6 & 7 = 3.57 $P < 0.01$
t between 6 & 8 = 3.17 $P < 0.01$

Note: *U* = unstimulated *in vitro*
A = trans dehydro androsterone glucoside (10 μg./25 mg. adrenals)
E = ethinyl estradiol (10 μg./25 mg. adrenals)
L = (Low) 0.015 I.U. ACTH/100 mg. adrenals.
H = (High) 0.045 I.U. ACTH/100 mg. adrenals.

tion was for only one hour. The glands previously inhibited by stilbestrol regained nearly all their ability to produce corticoids. The lower corticoid secretions of both control and experimental in Experiment e compared to their opposite number in Experiment d was probably due to lower substrate reserves in the third hour of incubation.

In Table 4 the effects of transdehydro androsterone glucoside and ethinyl estradiol on unstimulated and corticotrophin stimulated corticoid secretion are studied in one experimental design of 32 rats of eight litters and four females per litter. Ethinyl estradiol reduced adrenal steroid secretion while the transdehydro androsterone glucoside apparently increased it. This increase is probably due to an increase in α, β unsaturated ketonic, 240 mμ absorption by the hydrolysis of the 3-glucoside. This remains to be confirmed chromatographically.

The same effects were obtained in adrenals from male rats (Table 5).

From these experiments it is evident that the natural sex hormones have no immediate direct action on adrenal corticoid secretion. It is

interesting that the synthetic estrogens which can be very potent estrogenic compounds cause considerable inhibition of adrenal metabolism as do certain substituted natural estrogens.

Vogt (9) reported that hexestrol inhibited adrenal cortical secretion in the rat. She suggested that judging from the simularity in adrenal histological changes reported in the literature for all estrogens, the effect may not be peculiar to hexestrol. If this were so, the pituitary enlargement and adrenal hyperplasia that follow long-term extra estrogen treatment would arise then, not by direct stimulation to the pituitary but from increased pituitary secretion to compensate for the reduced adrenal cortical secre-

TABLE 5. TRANS DEHYDRO ANDROSTERONE GLUCOSIDE AND ETHINYL ESTRADIOL EFFECT ON *male* SPRAGUE-DAWLEY RAT ADRENAL CORTICOID SECRETION *in vitro*. RESULTS ARE EXPRESSED AS μG. CORTICOIDS/100 MG. ADRENALS

	1 *U+A*	2 *U+E*	3 *L*	4 *L+A*	5 *L+E*	6 *H*	7 *H+A*	8 *H+E*
	15.7	13.5	30.2	32.8	19.7	26.8	39.7	25.2
	16.4	15.2	29.4	28.0	22.8	31.2	28.7	21.7
	22.3	14.4	28.7	28.9	19.6	31.2	30.6	22.0
	26.0	13.9	26.4	29.9	22.2	28.4	39.7	24.6
Means	20.1	16.3	28.7	29.9	21.1	29.4	34.7	23.4

S.E. mean $\pm$ = 1.61

t between 1 & 2 = 1.67 $P > 0.1$
t between 3 & 4 = 0.53 $P > 0.6$
t between 3 & 5 = 3.40 $P < 0.01$
t between 6 & 7 = 2.34 $P < 0.05$
t between 6 & 8 = 2.64 $P < 0.02$

Note: Symbols are the same as those of Table 4.

tion. However, it is apparent from the work reported here that this is not the natural mechanism. The author previously reported (1) that both estradiol and testosterone given as subcutaneous depot doses caused functional and morphological changes in the anterior pituitary. Estradiol caused a greater pituitary enlargement than testosterone and a great reduction in alpha cells (growth hormone secreting) along with tremendous increases in beta and gamma cell (trophic hormone secreting) numbers and activity. A subcutaneous depot of estradiol was able to cause increased adrenal cortical secretion 16 hrs. later. The stimulation to the adrenal caused hyperplasia in short-term experiments. Continued stimulation sometimes led to decreased adrenal weight. However in all cases there was increased corticoid secretion on corticotrophin stimulation compared to the controls which would indicate a higher level of functional activity. The mechanism must be via the pituitary since the present paper shows no direct immediate adrenal effect of the natural estrogens.

The ultraviolet absorption method for corticoid estimation was used in this study because it is simple and precise. It is more suited to the many

quantitative measurements that have to be made in a preliminary screening of steroids that may affect adrenal function directly or via the pituitary. When an effect in the total corticoid output is found the differences in individual steroids can of course be studied with the aid of chromatography. At least 100 mg. of adrenal tissue is usually necessary to give sufficient steroids for satisfactory chromatographic identification.

Experiments are planned with other sex hormone derivatives both natural and synthetic of high and low potency. The effects of adrenal steroids both natural, synthetic and substituted on adrenal metabolism can, of course, also be studied with this system.

SUMMARY

The direct effect of some steroid compounds on total adrenal cortical secretion was studied *in vitro*. The natural sex hormones estradiol and testosterone had no immediate direct action on adrenal cortical function. A substituted estrogen, ethinyl estradiol and a synthetic estrogen, stilbestrol strongly inhibited adrenal corticoid secretion. The inhibition was reversible. The normal pathway for natural sex hormone effects on the adrenal cortex must be via the pituitary.

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PREPARATION OF URINARY EXTRACTS FOR THE ALDOSTERONE ASSAY: HYDROLYSIS AND EXTRACTION[1]

I. DYRENFURTH AND E. H. VENNING

McGill University Clinic, Royal Victoria Hospital, Montreal, Canada

THE early extracts of sodium retaining material from urine were prepared by acidification of the urine to pH 1.0 or 1.5 and immediate extraction with an organic solvent (2, 7). Later on, the acid hydrolysis was prolonged for 24 hours before the urine was extracted and considerably higher results were obtained (4, 6, 10). Also β-glucuronidase was used for hydrolysis and led to increased amounts of active material when compared to those obtained after immediate extraction of the acidified urine (1, 4, 8, 9, 10). These observations indicate that aldosterone occurs in the urine to the greater part in a conjugated form. The following experiments were carried out to establish conditions of hydrolysis, which would possibly lead to a maximum recovery of the aldosterone present in the urine.

METHODS

a) *Free fraction:* A 10-hour aliquot or more of a 24-hr. urine collection was adjusted to pH 6.0 and the urine extracted once with half and twice with a quarter of its volume of redistilled chloroform. The combined chloroform extracts were concentrated to approximately 400 ml. and washed 5 times each with 40 ml. of N/10 NaOH and 2 times with 40 ml. of water. Each washing was back extracted with 20 ml. of chloroform. The extract was brought to dryness under reduced pressure below 55° C.

b) *Acid hydrolysed fraction:* Urine or the urine remainder after the free fraction was removed was acidified to pH. 1.5 and allowed to stand at room temperature for 24 hours or longer before it was extracted in the described way.

c) *Enzyme hydrolysed fraction:* The pH of the urine or urine remainder after a or b was adjusted to pH 5.0, one Coleman buffer tablet pH 5.0 dissolved per 1 litre of urine and 200–300 units β-glucuronidase[2] per ml. urine were added. The mixture was incubated for 24 hrs. or longer. In some instances the neutral urine was boiled for 20 min. for sterilization before buffer and enzyme were added. When desoxyribonucleic acid was used 0.5 ml. of a 3% solution was added before the incubation for each 200 units enzyme present.

d) *Continuous extraction combined with acid hydrolysis:* The urine was acidified to pH 1.5 and placed in a continuous Hershberg Wolfe extractor. An equal volume of

Received August 6, 1956.

[1] These studies have been supported by grants from the National Research Council of Canada and the department of the Army Medical Research and Development Board, Washington, D.C.

[2] The β-glucuronidase preparation (Ketodase) was kindly supplied by the Warner-Chilcott Laboratories.

methylene chloride or chloroform was used as solvent. After 6 hrs. of extraction the extract was removed and replaced by fresh solvent with which the procedure was continued for 24 or 48 hrs. The combined extracts were treated as above.

All the extract residues obtained in these various ways were chromatographed in the Bush B-5 system and the eluate of the aldosterone zone was tested with the bioassay for sodium retaining steroids on adrenalectomized rats as described by Venning *et al.* (11).

The experiments were arranged in such a way that each group of extracts which were being compared were assayed against the same group of control and DCA-treated animals.

In most instances urines from pregnant women have been used to order to have urines with high aldosterone content.

EXPERIMENTS AND RESULTS

A) *Comparison of acid and enzymatic hydrolysis*

When the free fraction of aldosterone was extracted from the urine and aliquots of the remainder were treated for 24 or 48 hours either by acid or by β-glucuronidase hydrolysis, additional sodium retaining material was obtained in all acid and in most of the enzyme fractions. The amounts released by the acid hydrolysis exceeded those obtained after enzymatic cleavage (Table 1).

When both ways of hydrolysis were applied subsequently to the same

TABLE 1. EFFECT OF ENZYMATIC AND ACID HYDROLYSIS ON THE RECOVERY OF ALDOSTERONE (WHEN APPLIED SEPARATELY)

	μg. aldosterone/24 hr.		
	pH 6	β-glucuronidase	pH 1.5
Normal male			
1	<1.0	1.4	—
2	<1.0	4.3	—
3	<1.0	5.7	—
4	—	3.1	7.8
Normal female			
1	1.9	2.5	—
2	1.6	1.4	—
3	1.5	<1.0	—
4	—	<1.0	2.2
Normal pregnancy			
1	4.9	—	10.7
2	1.6	2.9	—
3	<1.0	4.6	8.9
4	3.9	—	9.6
Toxemia of pregnancy			
1	1.6	—	18.7
2	<1.0	—	3.7
Hypertension	—	<1.0	3.9
Adrenalectomy on Aldost.	1.7	4.6	13.3
Cirrh. of the liver			
1	—	1.8	5.2
2	—	2.6	5.3

urine after the removal of the free fraction it was found that acid hydrolysis following the enzyme led usually to considerably more additional material, whereas β-glucuronidase used as the last step released only small additional amounts (Table 2).

These results demonstrate that acid hydrolysis is more efficient than β-glucuronidase hydrolysis in releasing aldosterone from its conjugates. Attempts were therefore made to exclude possible bacterial or chemical inhibitors of the enzyme by either boiling the urine or by adding desoxyribonucleic acid to the incubation mixture. Forty-eight hrs. were allowed

TABLE 2. EFFECT OF ENZYMATIC ACID HYDROLYSIS ON THE RECOVERY OF ALDOSTERONE (WHEN APPLIED SUBSEQUENTLY)

	μg. aldosterone/24 hr.				
	1st step pH 6	2nd step β-gluc.	3rd step pH 1.5	2nd step pH 1.5	3rd step β-gluc.
Normal male					
1	—	3.1	5.2	7.8	3.6
2	—	—	—	2.2	<1.0
3	—	—	—	2.3	<1.0
Normal female	—	<1.0	2.5	2.2	1.1
Normal pregnancy					
1	—	6.6	19.4	—	—
2	—	—	—	23.0	9.2
3	1.6	2.9	19.5	—	—
4	—	16.9	8.2	19.2	1.6
5	2.5	2.3	20.9	—	—
6	5.2	12.5	4.3	—	—
7	2.2	10.4	12.5	—	—
8	1.8	11.6	3.7	—	—
9	1.6	2.9	15.6	—	—

for incubation. None of the procedures used increased the aldosterone value to the level obtained by acid hydrolysis during the same period of time. A subsequent acid hydrolysis for a further 48 hrs. released still more active material even when it followed after an initial hydrolysis at pH 1.5. The total amounts of aldosterone recovered from both samples which were enzyme-incubated in the first step were 12.3 and 14.1 μg./24 hrs., whereas with 96 hours of acid hydrolysis 20.8 μg. were obtained from the same urine, thus demonstrating that some active material has been destroyed at the elevated temperature of incubation (Fig. 1).

The amount of β-glucuronidase used in the above experiments was 200 units per ml. of urine. To exclude the possibility that this was insufficient a pregnancy urine was divided into two aliquots after the removal of the free fraction. On the one part enzyme hydrolysis was performed for 40 hrs. with 200 on the other part with 300 units of β-glucuronidase. Amounts of 2.9 and 2.3 μg. aldosterone/24 hrs. were obtained. Subsequent hydrolysis at pH 1.5 for 48 hours released additional 19.5 and 22.2 μg./24 hrs.

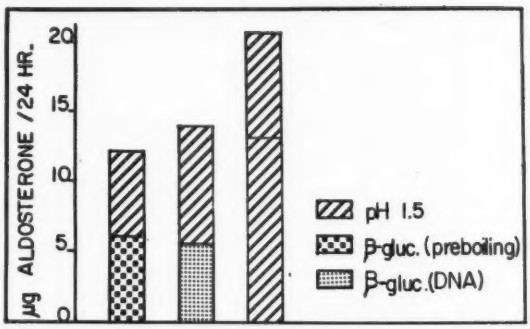

FIG. 1. Effect of enzymatic hydrolysis with DNA-treatment and preboiling of the urine.

These results clearly demonstrate that even a larger amount of enzyme is not capable of splitting the aldosterone conjugates still present in the urine. They suggest that the pH of the urine is an important factor for the hydrolysis of these conjugates. On the other hand the question arose, whether the amounts of aldosterone obtained after enzymatic cleavage are due to the specific activity of the β-glucuronidase or whether they are an effect merely of the unspecific incubation conditions, as time, temperature and buffering.

From two urines therefore aliquots were incubated at pH 5.0 and pH 7.0 without β-glucuronidase and at pH 5.0 with the enzyme. No increase of recovered aldosterone was observed after the incubation with the enzyme as compared to the control incubations (Table 3).

B) *Effect of the pH during acid hydrolysis*

Different aliquots of urine obtained from a patient with toxemia of pregnancy were adjusted to various pH-values, buffered and kept for 48 hrs. at 37° C. The recovered amounts of aldosterone were plotted versa the pH used during hydrolysis. The resulting curve showed that the hydrolysis becomes significant at the pH of 3 and reaches the maximum at pH 1.5. In this particular urine the recovery was less at pH 1.0 indicating some destruction of active material. A similar experiment carried out on urine obtained from a normal woman showed increasing amounts of aldosterone as the pH was reduced from 7 to 1.5. (Fig. 2).

TABLE 3. EFFECT OF β-GLUCURONIDASE AS COMPARED TO CONTROL INCUBATIONS

	µg. aldosterone/24 hr.		
	pH 5	pH 5 β-glucuronidase	pH 7
Normal female	2.7	2.4	2.4
Normal pregnancy	2.1	2.1	2.2

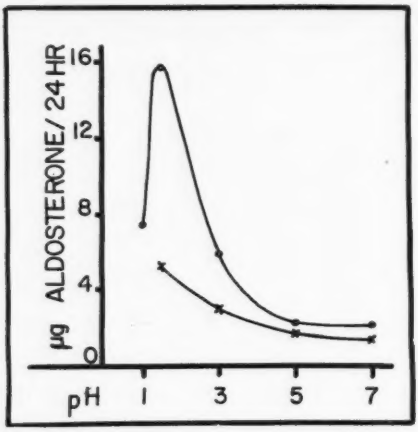

FIG. 2. Effect of the pH-value for the hydrolysis. x—x Normal female. o—o Normal pregnancy.

C) *Prolonged acid hydrolysis*

Two aliquots of a pregnancy urine were acidified to pH 1.5, allowed to stand at 25 and 37° C respectively and extracted repeatedly after hydrolysis for 24, 48 and 90 hours. At 25° C amounts of 10.7, 5.5 and 5.2 µg. Aldosterone/24 hrs. were obtained, thus showing that the hydrolysis was not complefe. At 37° C 10.2, 2.3 and 1.2 µg./24 hrs. were found. The total recovery was 21.4 for 25° C and 13.7 µg./24 hrs. for 37° C. Here again evidence is presented, that destruction of active material can take place in an acid medium especially over a longer period of time at elevated temperature.

Other urines acidified to pH 1.5 and extracted repeatedly at various time intervals also showed additional material when longer periods of time were allowed for hydrolysis. This time factor for the acid hydrolysis became most significant in the urine containing the highest amounts of aldosterone. In the urine of a normal male person the hydrolysis was practically completed after 96 hrs. (Fig. 3).

D) *Acid hydrolysis with simultaneous continuous extraction*

The above data suggested that measurable amounts of aldosterone are often left in the urine when it is extracted after 24 or 48 hours of acid hydrolysis and that furthermore destruction of some active material occurs, which becomes significant, when the urine is allowed to stand at the acid pH for several days.

Therefore continuous extraction of the acidified urine for 48 hours using methylene chloride or chloroform as solvent was compared to the extraction in separatory funnels. From four urines considerably higher results were obtained by the continuous procedure than by a careful extraction in

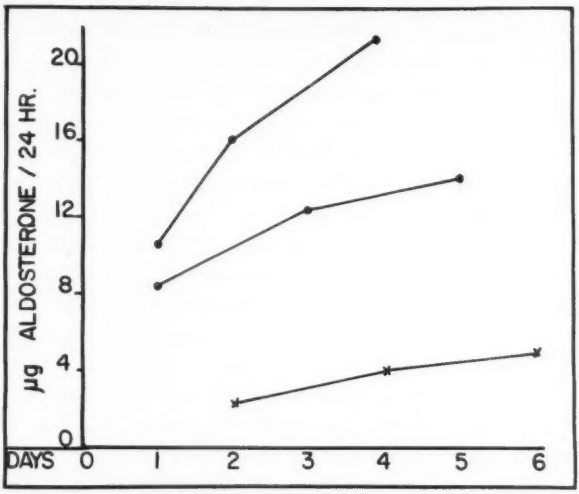

FIG. 3. Effect of prolonged acid hydrolysis. x—x Normal male. •—• Normal pregnancy.

the funnel. One urine did not show any significant difference between the two methods (Table 4). There was no apparent difference between the use of chloroform and methylene chloride as solvents. In one instance the continuous extraction was prolonged for 48 additional hours. Aldosterone determinations showed 2.5 μg. in this fraction whereas the extract of the first 48 hours contained 4.9 μg.

E) *Alkali washings*

The alkali washings of two extracts were assayed for their content of aldosterone. Whereas the mere back extraction of the sodium hydroxide with chloroform did not show any activity, it was possible to demonstrate small amounts of active material when the back extraction was performed after 24 hours of hydrolysis of the washings at pH 3, 1.5 or 1.0. Thus two urines, of which the extracts contained 8.5 and 10.2 μg. aldosterone per 24 hours gave values of 2.5 and 2.4 μg. aldosterone respectively for the hydrolysed washings.

TABLE 4. EFFECT OF CONTINUOUS EXTRACTION

	μg. aldosterone/24 hr.	
	Continuous	Sep. funnel
Normal female	4.9	5.2
Normal pregnancy		
1	21.0	8.5
2	18.7	13.0
3	10.1	6.6
Nephrosis	20.9	10.4

DISCUSSION

These studies show, that extraction of normal urine at pH 6 without previous hydrolysis results in the recovery of insignificant amounts of aldosterone. Furthermore the yield from all urines is considerably increased when the urine is allowed to stand at pH 1.5 for one or two days. This confirms earlier results (10, 11) and the observations of Neher and Wettstein (6) and of Axelrad, Luetscher *et al.* (1, 4).

The results are also in agreement with the findings of the above authors and of Gordon *et al.* (3) that acid hydrolysis yields larger amounts of aldosterone than hydrolysis with β-glucuronidase.

Although increased amounts of aldosterone could be obtained from the urine after β-glucuronidase hydrolysis as compared to those of the free fraction, wide variations have been observed in different urines regarding the percentage of total aldosterone which could be liberated by enzymatic hydrolysis. In one pregnancy urine β-glucuronidase released almost as much active material as the acid hydrolysis and from two other urines no aldosterone at all could be obtained with enzymatic hydrolysis, whereas the acid hydrolysis showed that measurable amounts were present. Whether these amounts obtained after hydrolysis with β-glucuronidase are due to the specific enzymatic activity appears to be doubtful. The above experiments on the urine of a normal and a pregnant woman did not show any increase in aldosterone recovered after incubation with the enzyme as compared to the control incubations. Luetscher (5) however, obtained from a urine of a patient with congestive heart failure a 50% increase in aldosterone in the enzyme incubated sample over the control incubation. Since it is known that β-glucuronidase can occur in the urine similar results from enzyme and control incubated urine portions do not exclude the possibility that there are small amounts of aldosterone present as glucuronides. On the other hand, the findings of Luetscher *et al.* may be interpreted as an action perhaps of sulfatase or of another enzyme which often accompanies β-glucuronidase preparations.

The amounts of aldosterone obtained from urine appears to be a function of the pH. The hydrolysis in experiments carried out at 37° C became apparent at pH 3 and reached a maximum at pH 1.5. At this temperature further acidification to pH 1.0 may cause destruction of aldosterone and lead to lower results.

The importance of the time factor for the acid hydrolysis was evident when the hydrolysis was extended over a period of 5 days. Small amounts of active material could still be recovered. Even continuous extraction for as long as 96 hours did not show completeness of aldosterone release.

From the above it seems apparent that the aldosterone occurs in the urine mainly in a conjugated form and that the major portion is not a glucuronide.

For the routine assay, hydrolysis conditions of pH 1.5 during 48 hours at 25° C have been chosen and continuous extraction with simultaneous hydrolysis is recommended for urines with higher aldosterone content.

SUMMARY

The conditions of hydrolysis for a maximal recovery of aldosterone from urines have been studied. Mild acid hydrolysis proved to be usually far more effective than β-glucuronidase hydrolysis. The degree of completeness reached appeared to be a function of the time, temperature and the pH at which the urine was allowed to stand. At room temperature the maximum recovery was generally obtained at pH 1.5. Some destruction of aldosterone in the acid medium took place which became significant at pH 1 and 37° C or at pH 1.5 and room temperature during longer periods of exposure to the acid. In some instances even after five days no completeness of the hydrolysis was obtained. The application of continuous extraction simultaneously with the acid hydrolysis, led to higher yields.

Acknowledgment

The authors wish to express their thanks to Mrs. Vessa Notchev for valuable technical assistance in these investigations.

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A COMPARISON OF THE EFFECT OF INANITION, CASTRATION AND TESTOSTERONE OF THE MUSCLES OF THE MALE GUINEA PIG[1,2,3]

CHARLES D. KOCHAKIAN, CAROL TILLOTSON AND JANE AUSTIN

Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma

CASTRATION results in a loss of weight of specific muscles of the male guinea pig (1). Since starvation also results in a loss in muscle mass (2–5), studies were conducted to compare the effects of inanition on the individual muscles in both the normal and castrated guinea pig and to determine whether testosterone could exert its growth stimulating effect during inanition.

PROCEDURE

Animals: Guinea pigs of the inbred Hartley strain were purchased at a body weight of 350 gm. (approximately 8 weeks-of-age) and maintained four in a cage in an air-conditioned room maintained at 22–23° C and with constant artificial light twelve hours per day. They were fed *ad libitum* the Rockland Guinea Pig diet containing vitamin C and in addition were given 30–40 gms. of fresh carrots and a 25 mg. tablet of vitamin C twice per week. When the animals reached 504 gms. (485–542), age 89 (83–92) days, they were divided into the respective groups according to weight. Castration was performed under ether anesthesia. The animals were autopsied 51 days later. The food was removed from the fasted groups 9 days prior to autopsy and the members of one castrated group were implanted subcutaneously with a pellet of testosterone[4] (approximately 15 mg.) at the time of removal of food and the animals of a second group were injected subcutaneously with 10 mg./day of testosterone propionate during the fasting period. A non-fasted group was simultaneously treated with the same dose of androgen. The animals were kept in individual cages during the fasting period. Another group of animals was castrated 9 days prior to autopsy to correspond with the fasting period.

At autopsy, the indicated organs were weighed on appropriate Roller-Smith torsion balances. The rest of the internal organs were discarded. The carcass was sealed in a plastic bag and frozen until time of dissection of the individual muscles.

The moisture and nitrogen content of the muscles were determined as previously described (1).

Received August 8, 1956.

[1] This investigation was aided under contract AT (40–1)–1741 with the United States Atomic Energy Commission and by an Institutional Grant from the American Cancer Society.

[2] These data were presented before the American Physiological Society at the Annual Meeting, April 16–20, 1956 (*Fed. Proc.* **15:** 187 (1956)).

[3] Technical assistance was provided by Don Cockrell and Louise Howell.

[4] The testosterone was generously provided by Syntex S. A. and was repurified by adsorption on alumina and recrystallization. The testosterone propionate (Perandren) was provided by Ciba Pharmaceuticals Inc.

RESULTS

Body Weights: The castrated guinea pigs showed a slightly slower rate of increase in body weight than the normals (Fig. 1) in agreement with the previous study (1). The withdrawal of food for 9 days produced identical percentage losses in body weight in the normal and castrated animals. The provision of testosterone during the fasting period had no detectable effect on the rate or total loss of body weight.

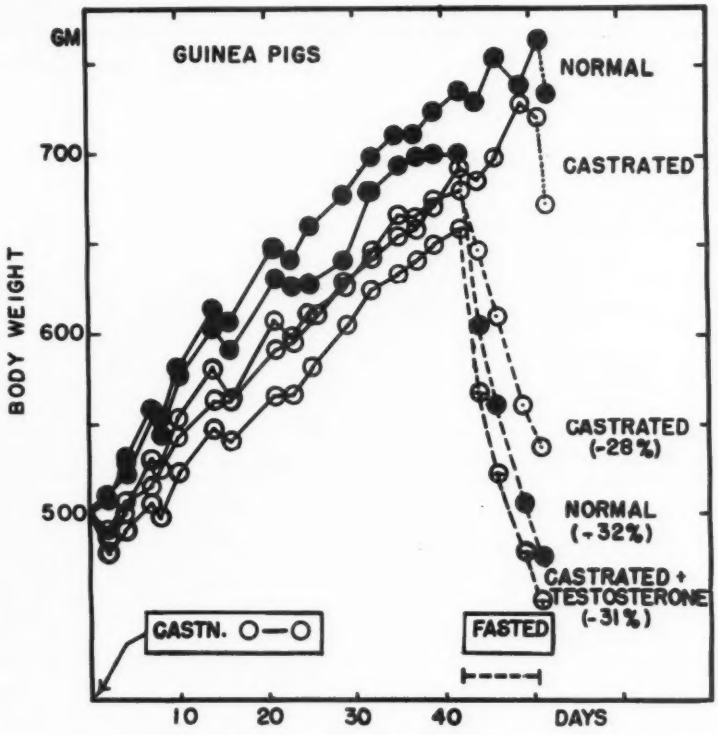

FIG. 1. The effect of castration and fasting on the body weight of young adult male guinea pigs.

Organ Weights: Castration did not significantly alter the weights of the various organs (cf. 1) or the retroperitoneal fat (Table 1).

The 9-day period of inanition produced a suggestive increase in size of the adrenal glands of the normal animals but was completely ineffective in the castrated guinea pigs. The report of a much greater increase in the adrenal cortex of young female guinea pigs after an acute or chronic fast has been attributed to an over-production of adrenocorticotrophin (6). The thymus was greatly decreased in size and the other organs decreased

in size to about the same degree or slightly less than the body weight (Table 1).

The average loss of retroperitoneal fat in the normal guinea pigs was not significant but that of the castrated animals including the treated groups was roughly proportional to the total loss in body weight.

Muscle Weights: Castration produced decreases in muscle weight (Fig. 2) similar to those previously reported (1). The greatest effect was in the retractor penis and the head muscles with decreasing effects in the neck and shoulder and back areas. The maximum decrease (retractor penis and temporal muscle), however, was not as great as that in the seminal vesicles and prostates. The muscles from the chest to the hind region differed in

TABLE 1. EFFECT OF A 9-DAY FAST AND TESTOSTERONE ON THE CARCASS AND ORGAN WEIGHTS OF THE MALE GUINEA PIG

Guinea pigs	Retroperitoneal fat (gm.) %	Carcass (gm.) %	Liver (gm.) %	Kidney (gm.) %	Heart (gm.) %	Thymus (gm.) %	Spleen (gm.) %	Adrenals (gm.) %
				Fed				
Normal[1]	(8.17) ±1.22	(542) ±14	(26.53) ±2.05	(4.926) ±0.235	(1.695) ±0.052	(1.389) ±0.136	(0.704) ±0.074	(0.400) ±0.023
C51[2]	− 4	− 8	+ 7	0	− 9	− 1	+ 3	− 9
C9[2]	−24	− 9	− 6	0	0	−21	+ 4	−22
C51+TP[4]	+ 7	−10	−18	− 7	−12	−17	+25	−10
				Fasted (9-days)				
Normal	−13	−31	−19	−10	−29	−65	−44	+21
C51[2]	−39	−29	−22	−21	−29	−72	−19	+ 2
C51[2]+T[3]	−57	−33	−22	−15	−31	−72	−33	+ 8
C51+TP[4]	−35	−33	−33	−16	−35	−33	−28	− 5

[1] Mean ± Standard Error.
[2] Castrated 51 and 9 days prior to autopsy.
[3] A pellet of testosterone was implanted subcutaneously at the initiation of the fast. Total absorbed 2.2 mg.
[4] The animals were injected subcutaneously with 10 mg./day of testosterone propionate for the 9 days prior to autopsy.

general less than 10% from those of the normal animals or changed roughly in proportion to the differences in carcass (or body) weight.

The withdrawal of food for nine days produced a decrease in weight of all the muscles of the normal animals to approximately the same degree with some exceptions (Fig. 2). The diaphragm and certain of the muscles of the hind leg decreased only about 20% while a few other muscles decreased as much as 40% and in one instance 46%. The nine day fast produced an effect on the muscles of the castrated animals similar to that in the normal animals. The fast produced only a small further decrease in those muscles which already had decreased as a result of castration.

Since fasting produced a decrease in the seminal vesicles and prostates and those muscles which responded to castration, a group of animals of the same weight as the normals was castrated 9 days prior to autopsy to correspond with the period of the fast. The decrease in the accessory sex organs was greater than that of the normal fasted guinea pig but the effect on the muscles was much less (Fig. 2).

Effect of Androgen: The administration of testosterone as a subcutaneously implanted pellet for the period of the fast was able to stimulate

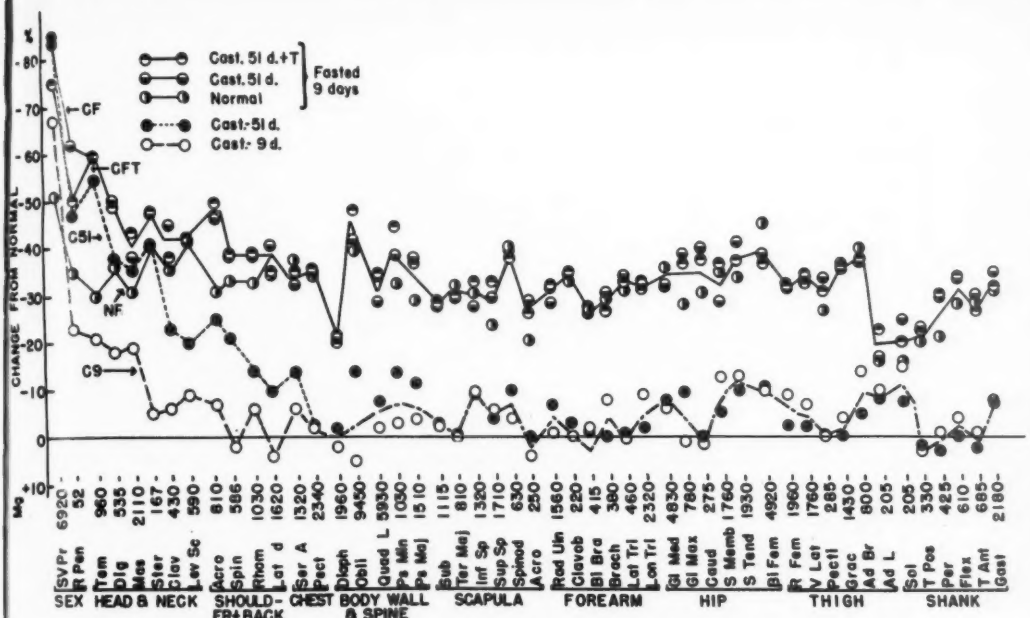

FIG. 2. The effect of castration, inanition and testosterone on the weight of the individual muscles of the young adult male guinea pig. Comparisons were made with the values of the normal group whose values are presented above. The full names of the individual muscles and the standard errors of the presented values are: (Seminal vesicles and prostates ±207); retractor penis ±3; temporal ±52; digastric ±26; masseter ±139; sternomastoid ±23; clavotrapezius ±18; levator scapulae ±28; acromiotrapezius ±55; spinotrapezius ±12; rhomboideus ±35; latissimus dorsi ±77; serratus anterior ±44; pectorals ±89; diaphragm ±172; obliques (internal and external) ±333; quadratus lumborum ±285; psoas minor ±52; psoas major ±51; subscapularis ±39; teres major ±41; infraspinatus ±70; supraspinatus ±60; spinodeltoid ±34; acromiodeltoid ±10; radial-ulnar complex ±75; clavobrachialis ±10; biceps brachii ±15; brachialis ±23; lateral head of triceps ±17; long head of triceps ±72; gluteus medius ±158; gluteus maximus ±61; caudofemoralis ±23; semimembranosus ±113; biceps femoris ±169; rectus femoris ±68; vastus lateralis ±36; pectineus ±25; gracilis ±71; adductor longus ±14; adductor brevis ±36; soleus ±8; tibialis posterior ±29; peroneus ±18; flexor hallucis longus ±28; tibialis anterior ±18; gastrocnemius ±81.

growth only in the sex tissues (Fig. 2). When a larger dose of androgen, testosterone propionate 10 mg./day for the 9 day period, was administered by subcutaneous injection, an increase occurred in not only the sex tissues but also in many of the muscles (Table 2, Fig. 3). The absolute increases were not as great in the fasted as the fed animals but the percentage increases were in some instances greater. Furthermore, two of the muscles, masseter and levator scapulae, were significantly increased in the fed but not the fasted animals.

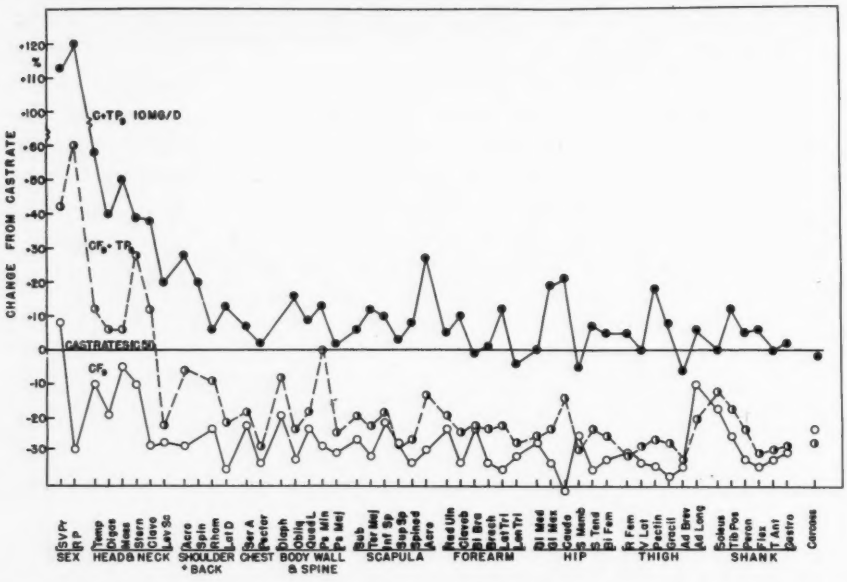

FIG. 3. Comparison of the effect of a high dose of testosterone propionate 10 mg/day for 9 days (TP_9) on the growth of the muscles of fasted (CF_9) and non-fasted (C) castrated guinea pigs. All of the animals were castrated 51 days prior to autopsy and the values are the differences from the castrated control group. See Table 2 for other details.

It is significant that the stimulation of the growth of the seminal vesicles and prostates was much less in the fasted than the fed guinea pigs and also, less than that with the low dose of testosterone.

Nitrogen (Protein) Concentration of Muscles: As in the previous study (1) castration did not alter the concentration of nitrogen (protein) even in those muscles which were greatly reduced in size (Table 3).

The withdrawal of food for 9 days resulted in a nearly proportionate decrease in the amount of nitrogen as in the weight of the muscles except that the decrease was consistently slightly less in the muscles of the normal animals and slightly greater in the castrated guinea pigs. Testosterone was without influence in these changes. The values for both the fasted and fed guinea pigs treated with the high dose of testosterone propionate were consistently lower for all of the muscles than those for the other groups of animals.

The values are presented as averages of the individual muscles for the respective areas to save space, but the results were consistent among the individual muscles but to different degrees.

Moisture Concentration of Muscles: Castration as in the previous study (1) had no effect on the moisture concentration of even those muscles which were markedly reduced in size (Table 4).

TABLE 2. COMPARISON OF THE MYOTROPHIC[1] PROPERTIES OF TESTOSTERONE PROPIONATE (T.P.)[2] IN FED- AND FASTED-CASTRATED GUINEA PIGS

	Fed			Fasted[3]		
	Control, (6)[4] mg.	TP (5) mg.	Diff. %	Control, (6) mg.	TP (4) mg.	Diff. %
Sex Tissues						
Sem. Ves. and Pros.	990	2130	+113	1080	1420	+39
Retractor Penis	28	48	+ 73	20	37	+85
Head and Neck						
Digastric	331	464	+ 40	269	350	+30
Masseter	1370	2046	+ 49	1305	1450	+11
Temporal	435	689	+ 58	391	493	+26
Clavotrapezius	330	457	+ 38	238	295	+24
Levator Scapulae	474	567	+ 20	348	368	+ 6
Sternomastoid	98	137	+ 40	88	126	+43
Shoulder and Back						
Acromiotrapezius	611	780	+ 28	435	571	+31
Spinotrapezius	461	552	+ 20	356	418	+17
Latissimus Dorsi	1461	1654	+ 13	955	1153	+21
Body Wall and Spine						
Diaphragm	1916	2805	+ 46	1542	1763	+14
Obliques	8113	9371	+ 16	5450	6261	+15
Psoas Minor	887	1012	+ 14	644	886	+38
Scapula						
Acromiodeltoid	251	319	+ 27	179	218	+22
Hip						
Caudofemoralis	277	336	+ 21	163	237	+45

[1] Only those muscles which were stimulated by TP are presented.
[2] TP was injected subcutaneously for 9 days at 10 mg./day.
[3] The guinea pigs were without food for the 9 days prior to autopsy.
[4] The values in parentheses indicate the number of animals per group.

The nine day period of fasting resulted in a slightly but consistently lower concentration of water in both the normal and castrated guinea pigs. Testosterone and testosterone propionate did not alter this trend.

Effect of Fasting on the Composition of the "Total Muscle Mass": The small difference in carcass weight between the normal and castrated animals was accompanied by a proportionate decrease in the total weight of

TABLE 3. EFFECT OF A 9-DAY FASTOON THE NITROGEN (PROTEIN) CONCENTRATION OF THE MUSCLES OF THE MALE GUINEA PIG[1]

Guinea pigs	Head and neck %	Chest %	Shoulder and back %	Body wall[2] and spine %	Scapula %	Forearm %	Hip %	Thigh %	Shank %
				Fed					
Normal	13.4	13.4	13.5	13.4	13.4	13.4	13.3	13.1	13.5
C51	13.4	13.1	12.9	13.3	13.4	13.5	13.4	13.4	13.3
C9	13.6	13.3	12.9	13.4	13.1	13.3	13.3	13.3	13.6
C51+TP	13.0	13.0	11.9	12.6	12.6	12.9	12.8	12.7	12.9
				Fasted (9-Days)					
Normal	14.0	13.5	13.1	13.8	13.9	13.6	13.8	13.8	13.9
C51	12.7	13.1	12.5	13.1	13.0	13.0	13.1	13.1	13.3
C51+T	12.8	13.1	12.8	13.5	12.8	13.2	13.4	13.5	13.3
C51+TP	12.4	11.6	11.8	12.8	12.6	12.8	12.8	12.5	12.9

[1] The values are the averages of the averages of the individual muscles of the respective areas (see Fig. 2).
[2] Diaphragm not included.

TABLE 4. EFFECT OF A 9-DAY FAST ON THE MOISTURE CONCENTRATION OF THE MUSCLES OF THE MALE GUINEA PIG[1]

Guinea pigs	Head and neck %	Chest %	Shoulder and back %	Body wall and spine %	Scapula %	Forearm %	Hip %	Thigh %	Shank %
				Fed					
Normal	77.8	77.4	75.7	76.5	77.2	76.4	76.3	76.9	75.7
C51	76.9	77.3	75.4	76.2	76.9	76.3	75.8	76.9	75.2
C9	77.4	76.9	75.6	76.7	76.2	75.9	76.0	77.1	75.9
C51+TP	77.3	77.3	76.1	76.5	76.4	76.5	76.5	77.2	76.2
				Fasted (9-days)					
Normal	75.5	75.5	73.5	75.8	75.4	74.2	74.6	75.6	74.4
C51	74.9	75.9	73.5	75.8	75.5	74.6	75.3	76.2	74.3
C51+T	75.2	76.7	74.3	76.6	75.6	75.0	75.3	76.4	74.6
C51+TP	74.7	74.4	73.5	74.6	74.1	73.5	74.4	75.0	74.0

[1] See Tables 1–3 for details.

the muscles which can be attributed to a loss in water and protein (Table 5).

The great decrease in carcass weight as a result of starvation also was accompanied by a proportionate decrease in muscle mass with a concomitant loss in water, protein and apparently fat ("rest") (Table 5). The loss in protein, however, was somewhat smaller in the normal and testosterone propionate treated groups. The change in the "rest" (fat?), which was determined by difference was variable but was always lower in the "fasted" than the corresponding "fed" group.

DISCUSSION

The effects of inanition on the body and organ weights of the guinea pig in general were similar to those previously reported (2–5) except that in a comparable and very careful study in the rat (4) only an 8% decrease in the "skin, musculature and skeleton" (carcass) was observed while more than a 30% decrease was reported in the other studies of a com-

TABLE 5. EFFECT OF CASTRATION AND FASTING ON THE COMPOSITION OF THE "TOTAL MUSCLES" OF THE MALE GUINEA PIG[1,2]

Guinea pigs	Carcass (gm.) %	Average of total muscles			
		Weight (gm.) %	Protein (gm.) %	Water (gm.) %	Rest[3] (gm.) %
			Fed		
Normal	(542)	(135.935)	(26.264)	(105.164)	(4.507)
C51	− 8	− 9.6	− 8.5	−11.3	+13.1
C9	− 9	− 4.5	− 2.9	− 5.9	+19.7
C51+TP	−10	− 2.2	− 6.7	− 2.7	+34.8
			Fasted (9-Days)		
Normal	−31	−34.4	−27.4	−36.6	−30.8
C51	−29	−35.9	−34.0	−37.4	− 8.9
C51+T	−33	−37.6	−35.3	−37.8	−35.0
C51+TP	−33	−29.5	−27.2	−32.1	+14.2

[1] See Tables 1–4 for details.
[2] The values are the per cent differences from the normal values which are in parentheses.
[3] Determined by difference and represents primarily fat.

parable nature (2, 3, 5). The decrease in total musculature of rats fasted for 9 (6–12) days was practically identical to that observed in the guinea pigs. Studies on individual muscles have been carried out only for 10 muscles and in the female rat fasted for 6 days. These results, however, indicated that muscles of the hind leg and the head did not decrease in proportion to the decrease in body weight. The results in the guinea pig, on the other hand, showed a marked decrease in the muscles of the head and a smaller decrease in those of the hind leg but the response of only the soleus was comparable with those in the rat. The more extensive studies in the guinea pig revealed other muscles which showed responses to fasting out of proportion to the change in body weight. The most significant of these in addition to the soleus are the adductor longus and the tibialis posterior of the hind region and the diaphragm. These variations from the decrease of the body weight probably is a reflection of the amount and lability of the reserve protein of the particular muscle.

The similar effect of castration and inanition and the lack of a summation of these two phenomena on those muscles which respond to castration indicate that the reserve protein in these muscles can respond equally to the two conditions. These muscles, however, must have a different mechanism for response to the two phenomena. The 9 day period of castration indicates that the effect of inanition on these muscles is not due to an "inanition hypophysectomy" and furthermore, the other muscles of the body respond to inanition in a manner comparable to these muscles but show no noteworthy response to castration. It should be noted that the loss of protein during inanition is a catabolic phenomenon presumably to provide energy and essential constituents for maintenance of life, while that after castration is due to the lack of the protein anabolic stimulus of androgen.

It is significant that the closely sex-linked tissues, the seminal vesicles and prostates and the retractor penis, were the only tissues which showed a significant response at the low dose of testosterone but at the higher dose of testosterone propionate many of the muscles also showed substantial increases. These changes indicate that essential tissue elements which are being mobilized to maintain life can be diverted by testosterone to the growth of specialized tissues. An analogous effect is noted during prolonged testosterone propionate treatment of rats fed *ad libitum* (6).

The proportionate decrease in all of the constituents of the muscles is of interest for it is generally believed (2, 3) that during fasting, the fat is mobilized prior to protein. The failure of a disproportionate decrease in the fat ("rest") of the muscles and the retroperitoneal fat suggests that the other fat depots, too, are not mobilized prior to mobilization of protein. Lasarew (cited in reference 2) noted, however, a greater loss of fat in the first few days of fasting followed by a greater loss of protein in the latter part of inanition in guinea pigs.

The rather generalized loss of protein during fasting in contrast to the

loss of apparently the same protein only in specific muscles after castration is in agreement with other conditions which preferentially mobilize material from specific tissues. The adrenal cortex mobilizes preferentially the protein from the lymphatic tissues (8, 9, 10) and to a lesser extent that of the carcass and liver (10). The thyroid on the other hand, is reported to mobilize primarily carcass protein (8, 9). Inanition, however, does not influence the size of the thyroid (2, 3), but apparently reduces the activity (6). Malignancy also is known to result in a mobilization of body protein apparently for the growth of the tumor (11) but whether this is a generalized effect as in inanition or occurs in specific muscles is not known.

SUMMARY

The removal of food for 9 days from young adult normal guinea pigs resulted in a decrease in body weight of 32%. The internal organs decreased as follows: liver 19%, kidney 10%, seminal vesicles and prostates 51%, heart 29%, thymus 65%, and spleen 44%. The individual muscles showed a decrease of 20–46% with most of the muscles decreasing about 35%. The diaphragm and muscles of the hind region (soleus, adductor longus and tibialis posterior) were in the low category with decreases of only about 20%. The decrease in the muscles of the head, neck and shoulder areas in the fasted snimals was similar to that in guinea pigs castrated for 51 days but greater than in those castrated for 9 days. The losses in protein and water of the individual muscles were roughly proportional to the losses in weight. Castrated animals showed a response to fasting similar to that of the normal animals. Only a slight summation of the effects of castration and inanition occurred. The administration of a small dose of testosterone to the castrated guinea pigs during the period of the fast produced a significant growth stimulus in only the accessory sex organs and the retractor penis, but a larger dose of testosterone propionate produced substantial increases in 12 skeletal muscles.

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A COTTON PELLET GRANULOMA ASSAY FOR LOCALLY ADMINISTERED ANTI-INFLAMMATORY DRUGS: 9 HALO, 21 DESOXY-CORTICOIDS

L. G. HERSHBERGER AND D. W. CALHOUN

Division of Biological Research, G. D. Searle & Co., Chicago, Illinois

MEIER, *et al.* (1), have demonstrated that foreign body granulomas are provoked in rats by subcutaneous implantation of cotton pellets and that administration of cortisone results in a diminution of granuloma formation. Shipley and Meyer (2) and others (3, 4) have developed assays for anti-inflammatory activity of systemically administered steroids which are based on these principles. Cotton granulomas also have been used to estimate circulating levels of endogenous anti-inflammatory substances during pregnancy and lactation (5, 6). Desaulles, *et al.* (7) and Stebbins and Stoerk (8) have studied the local effects of several substances in cotton granulomas by impregnating the cotton pellet with the test material before implantation. This cotton granuloma technique lends itself to the study of local steroid action because the same animal can bear both untreated and treated pellets, permitting a comparison.

Preliminary experiments in this laboratory using cotton pellets impregnated with corticoids indicated that cortisone and cortisol (17 hydroxycorticosterone) inhibited connective tissue growth around pellets which were implanted for 2 days, but they were much less active when the pellets were *in situ* for 6 days. This suggested that these compounds were absorbed too rapidly to be effective for as long as 6 days. This explanation received support when the relatively insoluble cortisol acetate was found to be extremely active for both 2 and 6 days. Further experimentation with cotton pellets impregnated with cortisol acetate has led to an assay method for local activity of long-acting anti-inflammatory steroids. This bioassay is the subject of the present paper.

MATERIALS AND METHODS

Male Sprague-Dawley rats averaging 175–200 gm. in weight were adrenalectomized and maintained on saline and Rockland Rat Diet throughout the experiment. The saline was supplemented with 0.5% glucose for 24 hours postoperatively. Cotton pellets[1] ranging from 5 to 8 mg. were weighed to the nearest 0.5 mg. on a torsion balance. A quarter cc. syringe was used to impregnate the pellets with test substance dissolved in 0.03 cc. acetone or a multiple of this volume if needed. Acetone is readily removed by evaporation. On the day following adrenalectomy, 4 pellets were implanted bilaterally

Received August 27, 1956.

[1] No. 3 cotton pellets, Richmond Dental Cotton Co., Charlotte, N.C.

into the loose subcutaneous connective tissue of the pectoral and dorso-lateral neck regions. Each animal was implanted with 2 treated and 2 untreated pellets, with the weight per pellet averaging 6 mg. in each pair.

Six days after implantation, the rats were sacrificed and each granuloma was dissected out and trimmed of the surrounding loose connective tissue. The pellets were dried to constant weight at 72° C and weighed to the nearest 0.1 mg. The granuloma response was expressed as net tissue weight per pellet for each rat.

RESULTS

Standard Curve: Study of Granuloma Response

Cortisol acetate was used at 6 different dose levels in preliminary studies with impregnated cotton pellets. Groups of 7 to 10 animals at a single dose

TABLE 1. EFFECT OF LOCAL CORTISOL ACETATE ON COTTON PELLET GRANULOMAS

(Net mg. tissue/pellet)

	μg. cortisol acetate/pellet					
	12.5	25	62.5	125	250	500
			Treated Pellets			
N	8	8	8	8	9	8
Median	9.85	6.65	6.90	6.80	3.00	3.35
Range	(8.5–13.1)	(4.4–12.2)	(4.6–8.1)	(4.5–8.6)	(2.6–5.2)	(2.7–4.6)
N	8	7	8	10	8	8
Median	8.05	8.20	6.40	5.95	5.40	2.45
Range	(6.1–9.8)	(6.8–12.3)	(5.4–9.9)	(4.7–12.0)	(4.1–7.0)	(2.0–3.5)
N		8				
Median		9.15				
Range		(6.4–12.0)				
			Untreated Pellets in the Same Animals			
Median	12.15	6.90	8.80	8.00	10.10	9.90
Range	(8.2–19.4)	(6.2–14.2)	(7.2–10.6)	(5.9–12.2)	(9.6–13.5)	(7.6–13.4)
Median	9.15	12.20	9.55	12.60	12.30	8.50
Range	(6.1–12.9)	(10.5–15.4)	(5.4–12.5)	(5.9–16.1)	(8.1–15.5)	(6.3–11.4)
Median		9.35				
Range		(7.4–11.8)				

were run at different times, for a total of 106 rats. Typical values for each group, represented by the group medians, are shown in Table 1. Increasing doses of cortisol acetate were associated with decreasing granuloma weight. Furthermore, the variation within groups appeared to decrease along with the typical values, as can be seen from the ranges in the table.

The correlation of variability with response level suggested that the logarithm of the net tissue weight might prove a satisfactory unit for calculation. After logarithmic transformation, the resulting group variances were homogeneous, as confirmed by the "maximum F-ratio" test[2]

[2] Details of this test were as follows. Among the 13 within-group variances calculated from transformed data, the largest and smallest had the values 0.0169 and 0.0040, respectively. Their ratio, 4.225, is well within the limits of random sampling variation for a body of data of this size.

(9). All further calculations were carried out using logarithms of granuloma weights.

Within-group variability of untreated log granuloma weights appeared equal to that of treated pellet values, giving a pooled standard deviation in log units of $s = \pm 0.096$. On taking the antilogarithm, this is translated into a 25% coefficient of variation.

Untreated pellet granulomas were correlated to some extent with deviations of the treated granulomas in the same animal. They did not appear to be affected one way or the other by treatment (Table 1), in spite of their implantation for 6 days in animals bearing treated pellets. It was possible by taking advantage of this correlation to increase the precision of the response. A response index for each animal was based on the treated granuloma weight appropriately adjusted for untreated granuloma weight as described below. This index, designated as y, has the following general form:

$$y = \log t + k \log u$$

where t and u represent treated and untreated[3] granuloma weights, respectively, and k is an adjustable factor. The ideal index was defined as one that would give maximal significance to the separation between mean responses at different doses. In the present data this condition was satisfied when the value of the arbitrary factor was $k = -0.3$, and the optimal response metameter was the index

$$y = \log t - 0.3 \log u.$$

This philosophy and calculation belong to the analysis of "discriminant functions," as discussed for example by Fisher (10). The index y is adopted as the measure of response in the following discussion. Its coefficient of variation is 23%, representing an increase in precision roughly equivalent to a 16% increase in number of animals.

Figure 1 shows the estimated dose-response curve for cortisol acetate. A straight line on the log-dose scale was fitted by least squares to the 13 group means. Its slope was $b = -0.278 \pm 0.039$. The responses in each group are represented in the figure by the 95% confidence interval for the group mean. It will be observed that the fitted line passes through each of these intervals. In theory, 19 in 20 of such intervals would bracket the true dose-response curve.

The analysis of variance for these 13 groups, based on the index y, is shown in Table 2. There appears to be a variation in mean response to a fixed dose on different weeks, as indicated by the highly significant "Between Weeks" mean square (rows 3 and 4). A test for non-linearity (row

[3] For convenience u was defined as net granuloma weight of untreated pellets, divided by 10 mg. The divisor, equal approximately to the over-all average of untreated granuloma weight, eliminates a constant bias from the adjusted response, y.

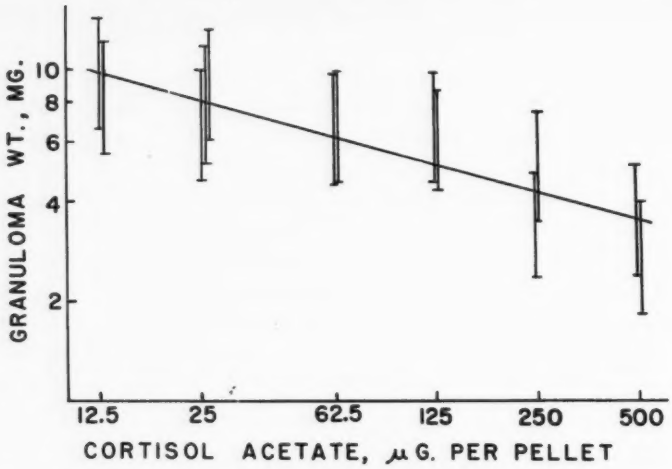

FIG. 1. Effect of local cortisol acetate on cotton pellet granulomas. Vertical bars represent the 95% confidence intervals.

2), using the "Between Weeks" term as error estimate, shows no significant curvature. Confidence limits shown in Figure 1 were conservative, being based on the "Between" rather than the "Within Weeks" variation. In effect, they make allowance for variation between groups run at different times which would be absent in comparing groups tested simultaneously.

Bioassay of Four Steroid Unknowns

The successful application of the present technique to bioassay depends on the attainment of a highly significant response slope. As the analysis indicates, this condition can be satisfied (Table 2, row 1). An experiment was carried out to confirm the utility of this response for the comparative assay of long-acting compounds.

Each of 5 steroids, four unknowns and a cortisol acetate standard, was tested at 3 dose levels in the ratios 1:2:4. One replication consisted of 2 rats at each dose level, with double this number for the standard. Rats were housed 2 to a cage, cage mates receiving the same treatment. This

TABLE 2. ANALYSIS OF VARIANCE FOR PRELIMINARY DATA: RESPONSE INDEX $y = \log t - 0.3 \log u$

Term	Degrees of freedom	Sum of squares	Mean square	Variance ratio
1. Slope*	1	2.4246	2.4246	60.01**
2. Non-Linearity	4	0.2549	0.0637	1.58
3. Between Weeks, Within Doses	7	0.2826	0.0404	5.11**
4. Within Weeks	93	0.7359	0.0079	

* Slope of dose-response line was $b = -0.278 \pm 0.039$.
** Highly significant, $P < 0.01$.

pattern was repeated 6 times on different weeks, giving totals of 36 rats on an unknown and 72 on the standard.

As a response metameter we used the index y that was described in the preceding section. Results of the assay are shown in Figure 2, where the mean response y is plotted for each dose, and dose-response lines are shown fitted for each compound.

Fitted lines for 4 of the 5 compounds were approximately parallel. The pooled slope for these 4, $b = -0.344$, was used for the dose-response lines

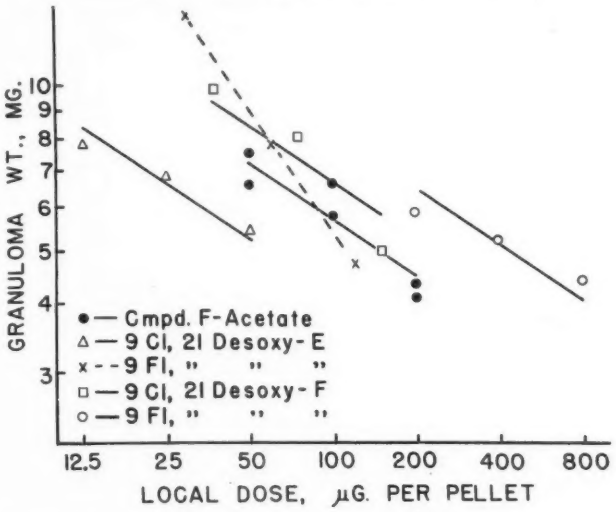

FIG. 2. Bioassay for anti-inflammatory properties of several 9α halo, 21 desoxy-corticoids against cortisol acetate standard.

in the figure and for calculation of potencies of three of the unknown steroids relative to cortisol acetate. This slope appears to be in good agreement with the earlier value for the standard alone, $b = -0.278$ (Table 2). The two slopes when tested showed no significant difference ($t = 1.32$).

Various checks on the validity of the assay as well as the estimates of experimental variation are shown in the analysis of variance, Table 3.[4] The relatively large source of variation between weeks (row 1) had been anticipated from the earlier studies. Separate interactions of weeks with the several treatment components have been isolated (rows 3, 5, 7 and 9) and do not appear to be negligible. This may indicate that the response metameter y could be improved by a further transformation. Meanwhile, for the present analysis, the significance of treatment comparisons would

[4] Duplicate groups receiving standard were analyzed as though they were separate compounds, so that in the analysis of variance there appear to be six materials. In Figure 2 two mean responses are shown for each dose of the standard, but a single line is fitted.

appear to be best judged against their individual weekly variations, rather than against the minimal variance between cage-mates (row 11). The response slope was highly significant (row 4′) compared to its weekly variation (row 5). Mean responses to compounds (row 2) differed significantly, indicating the choice of dose levels was imperfect, but this defect was not serious. In particular there was no evidence of general curvature (row 8), or of marked curvatures in dose-response lines for individual com-

TABLE 3. ANALYSIS OF VARIANCE FOR BIOASSAY

Term	D.F.	S.S.	M.S.	F
1. Between Weeks	5	1.9767	0.3953	
2. Compounds	5	0.9848	0.1970	5.80
3. Weekly Variation	25	0.8358	0.0334	
4. Average Slope	1	2.1776	2.1776	
5. Weekly Variation	5	0.2735	0.0547	
6. Between Slopes	5	0.4210	0.0842	4.70
7. Weekly Variation	25	0.4483	0.0179	
8. Average Curvature	1	0.0477	0.0477	1.43
9. Weekly Variation	5	0.1665	0.0333	
10. Residual	30	0.3732	0.0124	0.95
11. Cage-Mate Differences	106*	1.3887	0.0131	
Test for Parallelness, Excluding One Compound				
4′. Average Slope	1	1.2534	1.2534	22.91
6′. Non-Parallelness	4	0.1103	0.0276	1.54

* Degrees of freedom reduced from 108 because of two missing observations.

pounds (row 10), such as would be expected if extreme doses failed to lie within the approximately linear portion of the dose-response curve.

It is clear that the 6 fitted lines were not parallel (row 6). The first analysis of the slopes (rows 4–7) using a single pooled slope, gives a test for non-parallelness with 5 degrees of freedom that is highly significant. Selection of the compound with slope farthest from the others, 9α F, 21 desoxycortisone, and comparison of this one slope against the others, showed the non-conformity of this compound could account for some three-quarters of the "Between Slopes" sum of squares. An alternative analysis of slopes, excluding this one compound, shows a pooled slope term for the remaining steroids (row 4′) and a test for parallelness among them having 4 degrees of freedom (row 6′). Agreement among these latter slopes appeared to be sufficient to justify a discussion of relative potencies.

The relative potencies of the steroids, with 95% fiducial limits based on the cage-mate variance estimate of experimental error (table 3, row 11), were as follows:

Compound[5]	*Relative potency*	*Fiducial limits*
9α Cl, 21 desoxy-cortisone	2.57	1.85–3.52
9α F, 21 desoxy-cortisone	—	
Cortisol acetate	1.00	
9α Cl, 21 desoxy-cortisol	0.63	0.45–0.87
9α F, 21 desoxy-cortisol	0.34	0.25–0.47

[5] Synthesized by Drs. C. G. Bergstrom and R. M. Dodson, Chemical Research Division, G. D. Searle & Co.

The compound, 9α F, 21 desoxy-cortisone, gave responses approximately equal to the standard at doses near 100 μg. (Fig. 2). The slope for this compound differed significantly from all the others, so that strictly its relative potency was indeterminate.

DISCUSSION

The relative potencies in the table above show that the 9α chloro and similarly the 11-oxo configurations are associated with greater activity in this test than the corresponding 9α fluoro or 11-hydroxy analogues of these particular compounds. These activities might well be closely related to the solubility of the compounds in the tissue fluids as suggested by the difference between cortisol and cortisol acetate mentioned in the introduction.

The fiducial limits shown are descriptive of the particular experimental material used in this bioassay. They might be interpreted thus: "If these same 216 rats, over the same 6 weeks, had been randomly assigned to treatments in another way, the odds are 19 to 1 that any given calculated potency would have fallen within the stated limits." Extension of these results to cover different test conditions can be made using the estimates of weekly variation of compounds and slopes, shown in Table 3. Since these components are not estimated with any great precision, and since their causes are not clear to us, we feel it is more useful simply to present the above narrow fiducial limits for what they are worth.

There was no detectable effect of treatment on the untreated pellet granuloma weights. In Table 1, no correspondence appears between dose and the median untreated pellet responses. The existing correlation between treated and untreated pellet granulomas in the same animal argues for differences in responsiveness among animals, independent of treatment. No systemic factors that would explain this have been determined. Both the animal's initial body weight and the body weight change during the 6 days of treatment were found to be uncorrelated with variations in treated and untreated pellet granuloma weight.

The method described here provides a sensitive test for long-acting anti-inflammatory compounds. It is unique in that each animal bears treated and untreated pellets simultaneously. We feel that this method will be useful for the assay of other anti-inflammatory substances of this type and also that it offers possibilities for study of the mechanism of inflammation.

SUMMARY

A cotton pellet assay for prolonged anti-inflammatory activity of locally administered compounds was presented. The granuloma weights for control and treated pellets in the same animal were combined into an optimal response index. Several 9α halo steroids were assayed against a cortisol acetate standard. Relative potencies were estimated as follows: cortisol

acetate = 1.00; 9α chloro, 21 desoxy-cortisone = 2.57; 9α chloro, 21 desoxy-cortisol = 0.63; 9α fluoro, 21 desoxy-cortisol = 0.34.

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NOTES AND COMMENTS

QUALITATIVE DIFFERENCES IN THE PHYSIOLOGIC ACTIVITY OF THYROXINE AND ITS FORMIC ACID ANALOGUE

Lerman and Pitt-Rivers (1) have presented data suggesting that triiodothyroacetic acid may differ qualitatively from thyroxine in its physiologic effects. They noted that the administration of the compound to two patients with myxedema resulted in a fall in serum cholesterol levels without appreciable increase in the basal metabolic rate. This observation prompted us to investigate various analogues of thyroxine as regards their effect on cholesterol metabolism.

Tetraiodothyroformic acid, 4-(3′,5′-diiodo-4′-hydroxyphenoxy)-3,5-diiodobenzoic, acid, in which a carboxyl group is substituted for the alanine side chain of thyroxine is the subject of this report. The compound was described by Harington and Barger (2) and found to have thyroxine-like activity in amphibia and mammals by Frieden and Winzler (3). We have administered tetraiodothyroformic acid to albino rats and observed

TABLE 1. EFFECTS OF L-THYROXINE AND TETRAIODOTHYROFORMIC ACID ON THIOURACIL-INDUCED GOITRE AND CONCENTRATION OF CHOLESTEROL IN LIVER AND SERUM OF THE THIOURACIL-CHOLESTEROL FED RAT. VALUES GIVEN ARE THE MEAN AND STANDARD DEVIATION

Injected material	Daily amount	Number rats	Weight change	Thyroid weight	Liver cholesterol	Serum cholesterol
	mg./100 gm. body weight		gm.	mg./100 gm. body weight	mg./100 gm. liver	mg./100 ml.
			(+33)*	(6.5±0.9)*	(303±6)*	(72±5)*
0.9% Saline		7	− 6	16.7±2.6	1042±367	145±37
Sodium l-thyroxine	.003	7	+ 9	6.2±1.5	744±264	122±21
Tetraiodothyroformic acid	2.0	7	− 2	15.8±2.1	313± 36	112±13

* Mean values in our laboratory for rats fed the same basic diet but without added thiouracil or cholesterol.

a difference in its relative effect on thiouracil-induced goitre and on liver and serum cholesterol levels, as compared to thyroxine.

Three groups of male white rats (Holtzman) of 300 to 330 gm. weight were housed in mesh-bottomed cages and kept in an air-conditioned animal room at approximately 25 degrees C. During the experimental period of two weeks they were fed a diet consisting of Purina rabbit chow, 1% cholesterol, 0.5% thiouracil and 5% cottonseed oil, offered *ad libitum*. The animals in one group received daily subcutaneous injections of saline, in the second sodium l-thyroxine and in the third group tetraiodothyroformic acid. The latter two compounds were administered in 1% aqueous sodium bicarbonate, the volume injected being approximately 0.25 ml. At the end of 14 days the animals were anesthetized with intraperitoneal amobarbital sodium, blood collected by cardiotomy, and the liver and thyroid removed and weighed. Each liver was digested over night in 20% alcoholic potassium hydroxide. Total liver cholesterol and total serum cholesterol were determined by the method of Abell *et al.* (4).

The results are given in Table 1. It will be noted that at the dosage employed the thyroxine, as anticipated, completely prevented thiouracil-induced goitre while the

Received August 16, 1956.

tetraiodothyroformic acid had no inhibitory effect on goitre development. In contrast is the relative effectiveness of the two compounds in preventing accumulation of cholesterol in liver and serum. This is particularly apparent in regard to liver cholesterol, which in the tetraiodothyroformic acid injected animals approximates that of the normal rat fed neither cholesterol nor thiouracil.

These observations indicate that the substitution of a carboxyl group for the alanine side chain of thyroxine results in qualitative as well as quantitative changes in the physiologic activity of the compound. Tetraiodothyroformic acid, when compared to thyroxine, was found to exert relatively greater effect on cholesterol metabolism than in thiouracil-goitre prevention.

CHARLES H. DUNCAN, MAURICE M. BEST AND EARLE VAN HEYNINGEN
Department of Medicine
University of Louisville School of Medicine
Louisville
and
Lilly Research Laboratories
Indianapolis, Indiana

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PROTEIN HINDRANCE OF PREGNANEDIOL RECOVERY

It has previously been noted that urinary pregnanediol values are lower than normal in cases of toxemia of pregnancy (1, 2). In 1941 Bachman, Leekley and Hirshman observed that "Abnormal depression of excretion (pregnanediol glucuronide) was first noted at the time that proteinuria appeared, and was continuously associated with the latter phenomenon during the remainder of gestation" (3). The purpose of this study was to determine whether the presence of protein in urine would hinder the recovery of pregnanediol.

MATERIALS AND METHODS

Urines were collected from patients with normal pregnancies and pregnanediol was extracted by the Astwood and Jones technique (4) and determined colorimetrically in sulfuric acid. Human plasma was assumed to contain 7% protein and was added to suitable aliquots of the pregnancy urine. After the plasma was added, the mixture was allowed to stand overnight at room temperautre before starting the pregnanediol extraction.

RESULTS

Table 1 shows the results of an experiment in which the amount of pregnancy urine in each aliquot was kept constant and the amount and percentage of plasma protein was varied. From this table it seems that increasing the absolute amount of protein in the sample is an important factor in reducing pregnanediol recovery. Conversely, increasing the concentration of protein without altering the absolute amount had relatively little effect on pregnanediol recovery.

Six other similar experiments were done in which various types of protein were added

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Received August 25, 1956.

TABLE 1. PREGNANEDIOL RECOVERY AFTER ADDITION OF PROTEIN TO PREGNANCY URINE*

Added protein		Pregnanediol recovery		
Gm.	Per Cent	Mg.	Average, mg.	Per cent
0	0	13.6		
0	0	11.5	13.2	100
0	0	14.7		
1	.25	6.3		
1	.25	7.2	7.1	54
1	.25	7.9		
1	.5	9.6		
1	.5	9.6	9.9	75
1	.5	10.6		
1	.9	10.6		
1	.9	7.9	8.8	67
1	.9	8.7		
1	.9	8.2		
1	.9	8.4	8.1	61
1	.9	7.9		
2	1.0	6.5		
2	1.0	6.4	6.8	52
2	1.0	7.6		
4	.5	2.8		
4	.5	2.6	2.8	21
4	.5	2.9		
4	1.0	2.6		
4	1.0	2.8	2.7	20
4	1.0	2.6		
4	2.6	3.9		
4	2.6	3.6	3.8	29
4	2.6	3.9		

* This series of observations was made on a pooled collection of pregnancy urines. Values given are for six hour urine aliquots and would be representative of determinations on 24 hour urines containing about 50 mg. of pregnanediol and 4 to 16 gm. albumin.

to pregnancy urine. Two experiments were done with albuminous urine from patients with nephrosis, one with crystalline albumen, two with egg white and one with serum albumen as sources of protein. These experimental findings were all consistent in showing that the added protein significantly decreased the recovery of pregnanediol.

DISCUSSION

This study offers no evidence whether there is an adsorption or binding of the pregnanediol to protein or whether protein hinders the hydrolysis of the conjugated pregnanediol. However, earlier studies utilizing the Venning and Brown extraction for pregnanediol glucuronide also found lower values in toxemia of pregnancy (1, 3). Digestion or precipitation of the proteins prior to hydrolysis of the pregnanediol glucuronide should be explored as possible methods for more quantitative recovery of pregnanediol. Some such procedure will be necessary before it will be possible to determine whether the albuminuria, which commonly occurs in toxemia of pregnancy, hinders the recovery of pregnanediol. Estrogen excretion values have been reported lower in toxemic than in normal pregnancies (1) so the extraction of estrogens may also be hindered by urine albumin.

CONCLUSION

Protein added to pregnancy urine hinders the extraction of pregnanediol. This finding shows the need for new methods to determine the true excretion values for pregnanediol in toxemia of pregnancy.

JAMES T. BRADBURY AND THOMAS H. KENT

State University of Iowa
College of Medicine
Iowa City, Iowa

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PLASMA PROGESTIN LEVELS IN PROGESTERONE "ANESTHESIA"[1]

Selye has evaluated the ability of numerous steroid compounds to induce anesthesia in rats and mice and has placed progesterone high on the list, particularly when it is administered intraperitoneally (1). Chamorro reported that rats are seven times more sensitive than mice to the narcotic effect of a concentrated solution of progesterone in oil when the hormone is injected intraperitoneally (2).

Fifteen adult female mice of the CHI strain were ovariectomized. Fourteen to 30 days later they were injected intraperitoneally with 6 to 20 mg. of progesterone in 0.3 to 1 cc. sesame oil (13 mice) or with 0.5 or 1 cc. of oil only (two mice) (Table 1). The animals were observed for periods of from 43 minutes to 9½ hours and were then exsanguinated. The latter procedure was carried out by completing the anesthesia with ether and then exposing and dividing the carotid sheath vessels.

The blood sample and 0.05 cc. of 20% sodium citrate solution were centrifuged, and a measured volume of plasma was extracted and bio-assayed for protein "free" and "bound" progesterone equivalents according to procedures already described (3, 4). One *progesterone equivalent* (P.E) has been defined as equal to the activity of 1μg. of progesterone when the latter is assayed by the procedure of Hooker and Forbes in the absence of estrogen and other interfering factors (5). In two plasma samples, "free" levels of the hormone were moderately elevated (4.4 and 5.6 P.E./cc. plasma); in 13 samples, "free" levels were 1.3 P.E./cc. or less; in 7 of the latter, including the plasmas from the two mice receiving only oil, no activity was detected. "Bound" levels were determined in 11 plasmas; the levels ranged from 6.4 (in a single mouse accidentally injected subcutaneously) to 0.6 P.E./cc., with little or no activity being found in the "bound" fraction of the plasmas from the two control mice.

All animals were observed for unsteady gait, dormancy, ability to right themselves when placed on their backs, and ability to withdraw a paw when it was gently pinched. Behavior remained normal in the two controls, in one mouse injected with 6 mg., and in the one mouse accidentally injected subcutaneously (10 mg.) instead of intraperitoneally. Five other mice receiving 6–10 mg. appeared unaffected except for unsteady

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Received September 19, 1956.

[1] This research was supported by a grant from the National Institutes of Health, Public Health Service (RG-2342).

TABLE 1. "ANESTHETIC" EFFECT OF PROGESTERONE

Mgm. prog. injected	Interval[1] in mins.	Plasma level, P.E./cc.		Unsteady gait	Dormant	Righting reflex lost
		"Free"	"Bound"			
Oil only	143	<0.3	<0.5			
Oil only	186	<0.6	<0.6			
6.0	196	1.1	3.8	X	X?	
6.0	259	<0.5				
7.5	239	<0.9	1.9	X		
10.0	55	4.4	1.6	X	X	
10.0	167	0.5	<0.5	X		
10.0[2]	191	1.0	6.4			
10.0	201	<0.7	0.7	X	X	
12.0	567	<0.3	0.6	X	X	X
13.8	43	<0.4	3.3	X	X	X
16.0	145	0.4	1.3	X	X	X
20.0	120	1.3	0.9	X	X	X
20.0	151	<0.5	0.5	X	X	X
20.0	151	5.6	2.8	X	X	X?

[1] Period from injection to exsanguination.
[2] Subcutaneous injection.

gait. In all six mice receiving 12–20 mg., the gait was unsteady, the animals appeared dormant unless prodded repeatedly, and then moved only sluggishly, and they did not right themselves when placed on their backs. However, no one of the 15 animals failed to withdraw a paw which had been gently pinched. The degree of "anesthesia" did not appear to be correlated with the level of "free" or "bound" progesterone equivalents in the blood or with the time interval between injection and exsanguination.

These findings seem to indicate clearly that in castrate adult female CHI mice 1) the intraperitoneal injection in oil of 7.5–10 mg. of progesterone induced unsteadiness of gait, 2) that similar injection of 12–20 mg. of progesterone causes sufficient sluggishness so that the animal is unable or unwilling to right itself, 3) that even 20 mg. does not abolish a muscular response to a gentle pinch, 4) that anesthesia under these conditions is therefore incomplete, and 5) that the effects produced are a function of the size of the dose but are not proportional to the concomitant plasma levels of the hormone.

THOMAS R. FORBES

Department of Anatomy
Yale University
New Haven, Connecticut

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EFFECT OF A PRIMATE PITUITARY GROWTH HORMONE PREPARATION ON NITROGEN METABOLISM IN THE HYPOPHYSECTOMIZED RHESUS MONKEY[1]

The apparent ineffectiveness in man of currently available growth hormone preparations led us to an investigation of the actions of this hormone in the rhesus monkey. It was found that growth hormone of bovine and porcine origin when administered acutely or chronically to normal, hypophysectomized and sub-totally pancreatectomized monkeys at dose levels of from 5 to 20 mg. per kg. per day was without demonstrable effect on accretion of body weight, stimulation of the costochondral junction and various aspects of protein and carbohydrate metabolism (1, 2, 3, 4, 5). This report demonstrates the effectiveness of a crystalline monkey pituitary growth hormone preparation in inducing nitrogen retention in a hypophysectomized rhesus monkey in contrast to the physiological inertness of a similarly prepared sample of beef pituitary growth hormone.

MATERIALS AND METHODS

Two immature rhesus monkeys were placed in metabolism cages and fed a synthetic diet of known nitrogen content (6). The amount of diet offered to each animal was adjusted to approximate the daily food intake and kept constant throughout the experimental period. Water was provided *ad libitum*. At the end of the daily balance period (24 hours) urine, feces, spilled food and food remaining in the food cups were collected quantitatively and pooled. Nitrogen determinations on aliquots of the collection as well as on each new batch of diet were performed by the micro Kjeldal procedure with distillation. The amount of nitrogen retained or excreted was calculated by subtracting the total nitrogen recovered from the total nitrogen supplied.

Following a control period of several weeks the animals were hypophysectomized by the parapharyngeal route and nitrogen balance determinations continued until stabilization had occurred. At this time the administration of beef growth hormone (Armour lot #D674–010) was initiated and continued for 14 days at a dose level of 5 mg. per kg. per day. This experimental period was followed by a control period of 14 days. Purified monkey growth hormone was prepared by a slight modification of the procedure described by Wilhelmi (7). The material used is at the "crude crystalline" stage, which furnishes the greatest yield of a product which is essentially equipotent with a highly purified bovine growth hormone standard. This was administered at the same dose level (5 mg. per kg. per day) to one of the experimental animals for 14 days. The two hormone preparations were of approximately equal activity in terms of rat assay.

RESULTS AND DISCUSSION

The response of one animal to hypophysectomy and the administration of beef and monkey growth hormone in terms of daily nitrogen retention is illustrated in Figure 1. The vertical lines represent two standard errors of the mean. Incomplete collections were discarded and are not included in the calculations.

It may be seen from these data that the marked reduction of nitrogen retention occasioned by hypophysectomy of a young, actively growing monkey was not reversed or significantly modified by treatment with beef growth hormone. The other hypophysectomized animal presented an identical result. The administration of monkey pituitary growth hormone in equivalent quantities in terms of weight and potency (as measured in the rat), on the other hand, returned the daily nitrogen retention to pre-hypophysectomy levels. It is of interest that daily nitrogen retention returned to post-hypophysectomy levels after cessation of treatment with monkey growth hormone.

We have previously reported highly suggestive evidence of a similar dichotomy in physiological effectiveness of beef and pig growth hormone on one hand and monkey

Received September 29, 1956

[1] Supported by a grant from the American Cancer Society (EDC-18) and a grant from the National Institute of Arthritis and Metabolic Diseases (A-292), National Institutes of Health, U. S. Public Health Service and a grant (C2292) from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

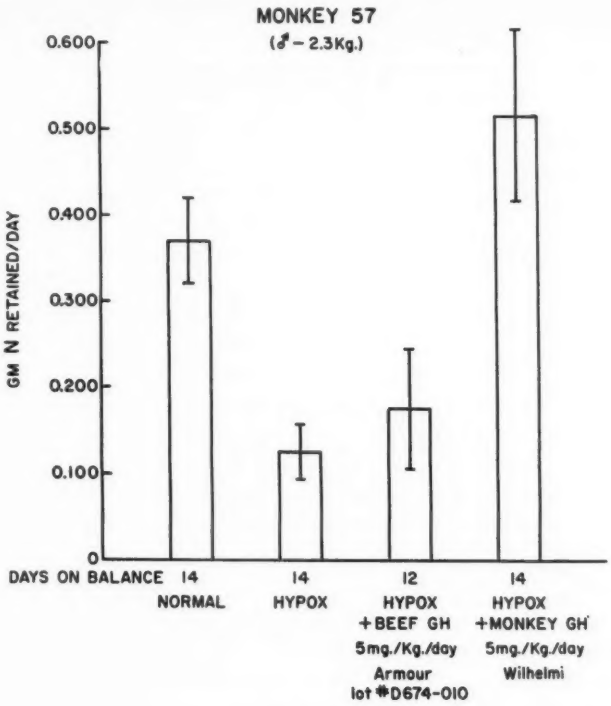

FIG. 1

growth hormone preparations on the other with reference to the repair of the deranged carbohydrate metabolism, and the atrophied costochondral junction following hypophysectomy in the rhesus monkey (5, 8, 9, 10).

These findings lead us to the conclusion that the apparent ineffectiveness of beef growth hormone in primates can probably be explained in terms of species specificity.

SUMMARY

Hypophysectomy markedly reduced the daily nitrogen retention in the growing rhesus monkey. The administration of beef growth hormone at a dose level of 5 mg. per kg. per day for 14 days did not significantly increase daily nitrogen retention in the hypophysectomized monkey. In contrast, monkey pituitary growth hormone administered to hypophysectomized monkeys at the same dose level for an equal period of time returned daily nitrogen retention to the pre-hypophysectomy level. The conclusion is reached that the apparent ineffectiveness of beef pituitary growth hormone in primates in contrast to the effectiveness of growth hormone preparations obtained from primate pituitary glands can probably be explained in terms of species specificity.

Acknowledgments

The authors wish to express their deep gratitude to Dr. D. A. McGinty of Parke, Davis and Co., Dr. Mary A. Root of Eli Lilly and Co., Dr. Joseph Seifter of Wyeth Institute of Medical Research, Dr. R. H. Barnes of Sharp and Dohme and Dr. C. A.

Bunde of Pitman-Moore and Co. for supplying the monkey pituitary glands. The beef pituitary growth hormone was generously provided by Dr. S. L. Steelman of Armour and Co. The Technical assistance of Mr. Edward Joseph is gratefully acknowledged.

ERNEST KNOBIL, RICHARD C. WOLF,[2] ROY O. GREEP AND ALFRED E. WILHELMI
Department of Physiology
Harvard Medical School
Boston 15, Massachusetts

Biological Research Laboratory
Harvard School of Dental Medicine
Boston 15, Massachusetts
and
Department of Biochemistry
Emory University
Emory University, Georgia

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[2] U. S. Public Health Service postdoctoral fellow.

THE ENDOCRINE SOCIETY

1957 Annual Meeting

The Thirty-ninth Annual Meeting of The Endocrine Society will be held in the Hotel New Yorker, New York, New York, Thursday, Friday, and Saturday, May 30, 31, June 1, 1957.

The Committee on Local Arrangements is Dr. Rulon W. Rawson as Chairman with Drs. Earl T. Engle, Joseph W. Jailer, Warren O. Nelson, and Martin Sonenberg as members of the Committee.

All Scientific Sessions will be held in the Hotel New Yorker. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 31, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Make reservations directly with the New Yorker, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Eleanor Venning, Royal Victoria Hospital, Montreal, Quebec, Canada, not later than February 1, 1957. *It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that these will be presented at the meeting*—in order that they may be of reference value and suitable for printing in the program and Journals of the Society. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   - Line 1. Title, not to exceed fifteen words.
   - Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be given after his name.
   - Line 3. Institution of origin and city in which institution is located.

3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter perfect, since there will be no opportunity for proof reading by authors.

1957 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Medal of The Endocrine Society

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. Dr. Carl R. Moore was the recipient in 1955 and Dr. Frederick L. Hisaw in 1956.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; 1949—Dr. George Sayers; 1950—Dr. Oscar M. Hechter; 1951—Dr. Albert Segaloff; 1952—Dr. Seymour Lieberman; 1953—Dr. Sidney Roberts and Dr. Clara M. Szego (Mrs. Roberts); 1954—Dr. I. N. Rosenberg; 1955—Dr. Jack Gross; 1956—Dr. Alfred M. Bongiovanni. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-four months of the date of the award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2.500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts

in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and The Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually for each individual and shall be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Medal of the Endocrine Society; the Ciba Award; the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *November 1* each year.

Proposals for appointment as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *November 1, 1956.*

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1, 1956.

Real Values For Endocrinologists

1. **HYPERPARATHYROIDISM.** By B. Marden Black, Mayo Clinic. Pub. '53, 127 pp., 24 il., (Amer. Lec. Endocrinology), Lexide, $3.75

2. **STUDIES ON TESTIS AND OVARY, EGGS AND SPERM.** Edited by Earl T. Engle. Pub. '52, 256 pp., 96 il., Cloth, $7.75

3. **LABORATORY AIDS IN ENDOCRINE DIAGNOSIS.** By Roberto F. Escamilla, Univ. Calif. Pub. '54, 152 pp., 16 il., (Amer. Lec. Endocrinology), Cloth, $4.75

4. **SIMMONDS' DISEASE: Extreme Insufficiency of the Adenohypophysis.** By R. F. Farquharson, Univ. Toronto. Pub. '50, 104 pp., 5 il., (Amer. Lec. Endocrinology), Lexide, $2.00

5. **HYPOTHALAMIC - HYPOPHYSIAL INTERRELATIONSHIPS: A Symposium.** Third Annual Scientific Meeting of the Houston Neurological Society, Texas Medical Center, Houston, Texas. Chairman, Hebbel E. Hoff. Compiled and edited by William S. Fields, Roger Guillemin, Charles A. Carton, all of Baylor Univ. Pub. '56, 176 pp., 79 il., Cloth, $4.75

6. **COMPARATIVE PHYSIOLOGY OF THE THYROID AND PARATHYROID GLANDS.** By Walter Fleischmann, Johns Hopkins Univ. Pub. '51, 85 pp., (Amer. Lec. Endocrinology), $2.50

7. **HORMONES AND BODY WATER.** By Robert Gaunt and James H. Birnie, Syracuse Univ. Pub. '51, 68 pp., 5 il., (Amer. Lec. Physiology), Lexide, $2.25

8. **HYPOGLYCEMIA AND THE HYPLOGLYCEMIC SYNDROME.** By A. J. Kauvar, Univ. Colo., and Martin G. Goldner, State Univ. N.Y. Pub. '54, 88 pp., 12 il., (Amer. Lec. Endocrinology), Lexide, $3.00

9. **SUBACUTE BACTERIAL ENDOCARDITIS.** By Andrew Kerr, Jr., Louisiana State Univ. Pub. '56, 368 pp., 16 il., (Amer. Lec. Internal Medicine), Cloth, $6.50

10. **THYROID FUNCTION AND ITS POSSIBLE ROLE IN VASCULAR DEGENERATION.** By William B. Kountz, Washington Univ. Pub. '51, 71 pp., 10 il., (Amer. Lec. Circulation), Lexide, $2.25

11. **SURGERY OF THE ADRENAL GLANDS.** By William Wallace Scott, Johns Hopkins Univ. Pub. '54, 136 pp., 60 il., (Amer. Lec. Abdominal Viscera), Lexide, $3.50

12. **TREATMENT OF TOXIC GOITER WITH RADIOACTIVE IODINE.** By Lindon Seed, Univ. Illinois; and Theodore Fields, Northwestern Univ. Pub. '53, 128 pp., 17 il., (Amer. Lec. Endocrinology), Lexide, $3.75

13. **HYPOTHYROIDISM: An Essay on Modern Medicine.** By Paul Starr, Univ. So. Calif. Pub. '54, 144 pp., 25 il., (Amer. Lec. Endocrinology), Lexide, $3.75

14. **STUDIES ON FERTILITY: The Proceedings of the Society for the Study of Fertility:** Volume VI (1954). Edited by R. G. Harrison. Pub. '55, 162 pp., 90 il., Cloth, $4.25

15. **STUDIES ON FERTILITY: The Proceedings of the Society for the Study of Fertility:** Volume VII (1955). Edited by R. G. Harrison. Pub. '56, 166 pp., 202 il., Cloth, $5.00

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